SYNTHESIS OF NOVEL MOLECULARLY IMPRINTED POLYMERS AND THEIR APPLICATION TO THE SOLID-PHASE EXTRACTION OF WATER-BASED MATRICES

Antoni Beltran Carbó

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Antoni Beltran i Carbó

Doctoral Thesis

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UNIVERSITAT ROVIRA I VIRGILI

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CERTIFIQUEM

Que la present tesi doctoral, que porta per títol "SYNTHESIS OF NOVEL MOLECULARLY IMPRINTED POLYMERS AND THEIR APPLICATION TO THE SOLID-PHASE EXTRACTION OF WATER-BASED MATRICES", presentada per ANTONI BELTRAN I CARBÓ, per optar al grau de Doctor amb Menció Europea per la Universitat Rovira i Virgili, ha estat realitzada sota la nostra direcció, a l'Àrea de Química Analítica del Departament de Química Analítica i Química Orgànica d'aquesta universitat, i que tots els resultats presentats són fruit d'experiències realitzades per l'esmentat doctorand.

I, per què consti, expedim aquest certificat a Tarragona, 31 de maig de 2010.

Prof. Francesc Borrull i Ballarín Prof. Rosa Maria Marcé i Recasens

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Després d'aquesta aventura és el moment de fer balanç de totes les coses bones i de les millors que han passat per a poder arribar finalment a bon port i que han fet que aquesta aventura sigui única, irrepetible i inoblidable. Les coses bones m'han ajudat a aprendre i créixer una mica més i, pel que fa a les millors, no haguessin pogut passar sense l'ajuda de les diferents persones que he tingut al costat al llarg d'aquest temps.

Entre elles, primerament vull agrair al Prof. Francesc Borrull i la Prof. Rosa M Marcé l'oportunitat de realitzar la tesi doctoral sota la seva direcció dins del "Grup de Cromatografia i Aplicacions Mediambientals". Gràcies per totes les tardes que hem passat asseguts parlant i pel bons consells que m'heu donat per a tirar endavant aquest projecte; intentaré treure'n l'essència i fer-los servir en situacions similars.

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Un apunt especial es mereix la Núria ja que, si bé també és integrant del mateix grup, amb ella he tingut el plaer de poder treballar no només al laboratori del grup sinó també al de la Universitat d'Strathclyde i de tenir discussions molt interessants no només sobre ciència.

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També a totes aquelles persones que es van incorporar després de mi i amb les quals hem passat tantes bones estones junts, no només al laboratori. Gràcies Jasper, Irene, Laura, Sílvia, Vero, Jordi, Dominika, Igor, Noelia, Blanca i Maria pels bons moments i les rialles que hem compartit junts.

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En aquest apartat tampoc hem puc oblidar dels companys amb qui comparteixo el dia a dia actualment, ja que ells fan que les hores de feina siguin molt més productives i divertides; sobretot quan ens reunim per a alliberar tensions. Entre ells, vull destacar als *metabolomiquets*; la Mariona, el Miguel i la Sara i al nostre director, el Xavier, per tot el seu recolzament en aquest tram final de la tesi.

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A tots vosaltres,

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Due to the wide acceptance of molecularly imprinted polymers in regard to selective extractions, the aim of this thesis is to develop novel imprinted materials targeted for different compounds included within the so-called group of organic emerging contaminants.

Another objective is to exploit different synthetic protocols and asses the different advantages and drawbacks that those approaches bring to imprinted polymers.

The molecularly imprinted polymers synthesised will be applied to the extraction of the molecule used during the synthesis of the polymer from water-based matrices.

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1. Introduction

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Due to the growing interest in determining many different compounds at various concentration levels in highly complex matrices, many different techniques have been developed. The most widely used techniques for separating and determining such compounds are gas and liquid chromatography, although many other techniques are also being used. In most cases, the sample of interest is a highly complex matrix in which the analytes of interest are at very low concentration levels. In these cases, a sample pre-treatment stage prior to the instrumental determination of the analyte of interest is required. The sample pre-treatment stage has two main objectives: to preconcentrate the analyte of interest in the sample in order to enable a lower concentration to be quantifiable, and to discard some other components also present in the sample which makes it difficult to quantify the analytes of interest. This simple method enables the quantification of the analytes present in a given sample by the most commonly-used detection systems - such as UV or fluorescence for liquid chromatography - at concentration levels that would not be possible if this pre-treatment stage had not had been performed. However, when the concentration of the analyte is too low for the commonly used detection systems, a more sensitive detector, such as mass-spectrometry (MS), is required which also enables the confirmation of the presence of the compound in the sample.

There are several techniques to perform the preconcentration of liquid samples. The first used was liquid-liquid extraction but, due to the large volume of solvents required for performing this extraction, this technique is being replaced in many applications. The most widely used extracting methods are those based on the retention of the analytes of interest on to a solid support. In these methods, a solid support is brought in contact with a liquid solution and the analytes present in the solution are retained on to the solid sorbent. There are several techniques developed to meet this goal, such as stir-bar sorptive extraction (SBSE), solid-phase microextraction (SPME) or matrix solid-phase dispersion (MSPD) but the most widely used is solid-phase extraction (SPE). With these techniques, the use of organic solvents is dramatically reduced and high preconcentration factors of the analyte of interest are achieved.

In SPE, as stated previously, the analytes present in a liquid matrix are retained on to a solid sorbent and further desorbed for their quantification. The first sorbents used in SPE extraction protocols were silica-bonded and carbonaceous sorbents.

Silica-bonded sorbents are obtained by chemically bonding different groups onto silica particles and, depending on the groups attached to the silica particles, these sorbents can be used for extracting analytes of interest from aqueous or organic solvents. When those sorbents are to be used for extracting analytes of interest from organic solvents, the groups attached to the silica particle were cyanopropil, aminopropyl or diol functional groups, whereas when the analytes are present in aqueous matrices, the mostly used groups were octadecyl (C_{18}), octacyl (C_8), ethyl (C_2), phenyl and cyclohexyl. However, due to the nature of these sorbents, the main drawbacks when using them were the low recoveries for polar compounds, the

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instability at extreme pH (due to the breakage of the bond between the silica particles and the groups) or the presence on the sorbent of residual silanol groups.

The other group primarily used as SPE sorbents were carbonaceous materials, such as graphitised carbon blacks and porous graphitic carbon. Those sorbents have very interesting physical properties - such as very robust chemical, mechanical and thermal properties - as well as very high adsorption and capacity despite the fact that their surface areas are not very high. However, the main drawback of those sorbents is the excessive retention of some compounds which, in some cases, remained irreversibly bonded onto this material [1].

To overcome the drawbacks of both silica-based and carbonaceous sorbents, polymeric sorbents were developed [2,3]. The main improvement that polymeric sorbents offer over silica-based sorbents is their stability thorough all of the pH range. The main improvement over carbonaceous sorbents is the possibility for establishing reversible interactions with all of the analytes present in a sample, thereby facilitating the uptake and release of the analytes onto the sorbent. Polymeric sorbents are mainly obtained by copolymerisation of styrene and divinylbenzene (DVB) and retention of the compounds on to this sorbent are through both hydrophobic and Л-Л interactions. Those sorbents have been widely accepted and used in many different applications and are actually the most widely used sorbents in SPE. A good example of its popularity is the large number of commercially available ones.

The next step after polymeric sorbents were highly cross-linked polymers. These were developed in order to increase the capacity of polymeric sorbents. To increase the capacity, an increase in the ratio of DVB/styrene was adopted in order to increase the number of Л-Л interactions. However, this approach has a limitation because once a certain concentration of DVB has been reached, the cross-linking efficiency decreases because of steric impediments and, therefore, no improvement in the surface area of the polymer is obtained [4].

To overcome the drawback of highly cross-linked polymeric sorbents when increasing surface area, Davankov and Tsyurupa [5] developed a new polymerisation technique in the 1970s to deliver what is known as hypercrosslinked polymeric sorbents. This technique is based on the cross-linking of preformed linear (or slightly cross-linked) polystyrene *via* the Friedel-Craft reaction. In this way, high surface areas (up to a four fold increase compared to polymeric sorbents) can be obtained [1] and, therefore, the sorbent capacities are higher.

Despite the big improvements to sorbent capacities and preconcentration of the samples obtained in all of the different approaches described previously, retention of the compounds on all these sorbents was mainly through hydrophobic or Л-Л interactions and, therefore, the most polar compounds were not retained on these kinds of cartridges.

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At the same time as hypercrosslinked polymers were developed for improving the surface area of polymeric sorbents, a different trend for synthesising these sorbents for retaining polar compounds was also developed. These sorbents are known as hydrophilic sorbents and are based on polymeric sorbents in which some polar functionalities have been incorporated, giving them the ability to retain polar and non-polar compounds.

The method of obtaining such polymers can be done either by co-polymerising a polar monomer with a cross-linking agent (generally DVB) or by chemically modifying already existing commercially available sorbents with polar functionalities.

Regarding copolymerisation of polar monomers, our group has extensively reported on the production of these kinds of sorbents and has successfully applied them in extracting phenolic compounds and polar pesticides from water samples [6]. Even though, there are some commercially-available SPE sorbents obtained by co-polymerising polar groups and DVB such as Amberlite XAD (Rohm & Hass) or Oasis HLB (Waters) amongst others.

The second approach for obtaining hydrophilic polymers is by a chemical modification of already existing hydrophobic sorbents. This approach was first described by Fritz *et al*. [7,8] by incorporating sulfonic, hydroxymethyl and acetyl groups in styrenic resins. In the late 1990s, our group reported on the modification of commercially available sorbents with acetyl [9] or benzoyl [10] groups, among others, and the modified sorbents showed improved extraction efficiency for most polar compounds compared with the non-modified ones. Nowadays there are some commercially-available modifies sorbents such as solute ENV+ (IST) or Strata-X (Phenomenex) amongst others.

Therefore, the use of the two different approaches for obtaining hydrophilic sorbents has been widely accepted and nowadays there are several different commercially available sorbents for performing extraction of polar compounds [11].

It is possible to take the extraction of polar compounds one step further by using sorbents which display a certain degree of selectivity for retaining polar compounds sharing similar acid or basic characteristics. Those sorbents are known as mixed-mode sorbents. In this case, the sorbents have a polymeric structure incorporating ion-exchanger group pendants on their structures and their selectivity can be tuned by carefully switching the pH of the solvent used in either the loading or eluting stages, as was the case of the extraction of triazine metabolites from soils [12]. This feature enables a clean-up of the sample prior to the elution of the cartridge to be performed, thus obtaining cleaner extracts. Another interesting feature of these sorbents is that they can retain compounds by both hydrophobic interactions through the structure of the sorbent and ionic species through the ionic groups incorporated during the synthesis of the polymer. Due to the improvement in extraction of polar compounds, mixed-mode sorbents are also commercially available, such as Oasis

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WAX and Oasis WCX (Waters) or Strata–X–C amongst others, although many different research projects are on-going [13].

A common feature among all the sorbents described so far is that none of them are able to perform selective extractions for a single compound or very close related to. In all of these sorbents, the analytes retained are those that show complimentary functionalities to the polymer, whereas the rest of the compounds are eluted. Therefore, all the analytes retained on these sorbents share similar characteristics. It is therefore very difficult (and sometimes even impossible) to discern between compounds sharing a similar polarity that have completely different sizes and shapes.

When aiming to perform highly selective extractions, the first sorbents used were immunosorbents (IS). IS are sorbents which enable very selective extraction indeed and they have been used extensively in highly selective applications [14,15]. These sorbents are based on the antigen-antibody principle and, therefore, when aiming to detect a particular antibody in a matrix, its antigen is immobilised on a solid support and, once a matrix is percolated through this sorbent, the analyte of interest is retained by highly-selective interactions. However, since both antigen and antibody are proteins in nature, the use of these sorbents is limited enormously. On the one hand, in order to obtain the antigen required for the intended application, this antigen must be produced '*in-vivo'*, which is both difficult and expensive to achieve. On the other hand and also due to their proteic nature, in order to not disrupt the proper activity of neither the antigen nor the antibody, the sample conditions for extracting the analyte of interest must also be compatible with the optimal conditions of those proteins. Therefore, no harsh conditions, such as an extreme pH level or organic solvents, can be used in these protocols because they would deprotenise the antigen immobilised on the sorbent, leading to no retention at all of the target analyte.

The application of IS for selective extraction is very limited for a number of reasons. These include the high costs associated with producing IS, the very strict conditions required for their proper use, the limited number of times that IS can be reused as well as the low number of molecules that can be extracted using this method.

To overcome the drawbacks of IS when performing selective extractions as well as to make the most of all the advantages that polymeric sorbents can bring, a new trend for using highly selective polymeric sorbents appeared in the mid-1990s. These sorbents are known as molecularly imprinted polymers (MIPs). Even though MIPs appeared in the early 1970s, it was not until Sellergren [16,17] used them for the first time to extract pentamidine from urine samples that they became increasingly used for SPE. The use of MIPs as sorbents in SPE has led to this SPE protocol being known as molecularly imprinted solid-phase extraction (MISPE).

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Due to the polymeric nature of MIPs, these sorbents try to emulate the high selectivity obtained when using IS combined with the high cost-effectiveness of polymeric sorbents.

Therefore, polymeric sorbents which can be reused many different times with no losses in their performance, which can work with all of the pH ranges and which can withstand any experimental conditions (even organic solvents) are easily produced. However, in regard to selective extractions, the MIPs and immunosorbents do not need to be excluded from each other. They can be used in a complimentary way. This complimentarity lies with the target molecules to be extracted in any of these techniques. For immunosorbents, the target molecules are generally large and highly functionalised and can adopt many different configurations. On the contrary, in the case of MIPs, the molecules normally used are rather small and rigid. Therefore, many different molecules can be selectively extracted by combining the two techniques.

Because MIPs offer many advantages over all of the other available sorbents these days, their use has been widely exploited in many different fields and many new research projects are on going.

Although MIPs have been used in several applications described in literature including sensors [18,19], capillary electrophoresis [20], catalysts [21-23], stationary phases in liquid chromatography [24,25] or enantiomeric separation [26] amongst others, the one in which MIPs have been mostly accepted is as sorbents in solid-phase extraction (SPE) protocols.

1.1 Synthesis of molecularly imprinted polymers

MIPs are tailor-made sorbents specially designed for the analyte of interest. They are synthesised by copolymerising the suitable monomer(s) and cross-linking agent in the presence of the molecule to which the sorbent is intended to be selective for and this molecule is known as the template molecule.The high degree of selectivity arises because, during the synthesis of the MIP, the molecule that the MIP is intended to be selective for is admixed with the prepolymerisation mixture and polymerisation proceeds in the presence of this molecule.

Although the first reference to a polymerisation in the presence of a template molecule dates back to 1931, when Polyakov synthesised a silica matrix and wrapped a template molecule inside [27], it was not until 1972 when Ali Sarhan *et al*. reported the first imprinted polymer as we know them today [28]. From 1972, several papers were published in this field but it was not until 1983 when Vlatakis *et al*. [29] published a paper that the use of MIPs in many different applications took off. However, it was not until 1994 when Sellergren reported the first use of MIPs as sorbents for SPE [17].

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The synthesis of MIPs starts in the same way as with any other kind of polymerisation: all the monomers involved in the polymerisation process are mixed together and then left to polymerise. However, selection of the monomers involved in the polymerisation process is crucial for the MIP to have the desired selectivity. In the synthesis of MIPs, all the monomers used in the polymerisation process have a very well-defined role and are known as functional monomers and cross-linking agents.

The general procedure for obtaining MIPs is to mix the template molecule with the functional monomer. The functional monomer must always have complimentary functionalities to the template molecule because, once the MIP is obtained, the functional monomer is the part responsible for the selective retention of the analyte on to the MIP. Due to this complementarity, a complex template molecule-functional monomer is formed by self-assembly of those molecules when the functional monomer and the template molecule are mixed together. This complex is essential for a proper recognition of the target molecule by the MIP. Afterwards, a cross-linking agent is added. Its function is to link the polymeric chains growing in the solution and to deliver the mechanical properties for the intended application.

As in any polymerisation process, an initiator agent must be introduced into the solution to induce polymerisation after all of the compounds involved in the synthesis of the MIP have been added. This agent normally has an unstable structure that, once exposed to either UV radiation or heat, easily decomposes into radicals, which are responsible for starting the polymerisation process.

Depending upon the different experimental conditions in which this polymerisation process occurs, the polymer finally obtained might have dramatically different shapes and degrees of flexibility. Generally, the degree of flexibility is inversely related to the degree of cross-linking agent used in the polymerisation process. The cross-linking agent is a monomer used in the polymerisation process whose function is to link different polymeric chains as they grow in the solution, thus impairing rigidity to the polymer once it is formed.

Since their main application in the analytical field are as sorbents in SPE protocols, MIPs need to have very high mechanical properties. This is achieved by using an excess of cross-linking agent to ensure a robust polymeric material with good mechanical properties.

Once the polymer is obtained, the template molecule is extracted, leaving behind different cavities that are not only complimentary in size and shape to the template molecule but also have complimentary functionalities to this molecule. The functionalities arise from the fact that the template molecule and the functional monomer establish a complex prior to the polymerisation process. Once the polymerisation process is finished, the functional monomer remains attached to the polymer, whereas the template molecule is easily removed. Therefore, cavities within the MIP are complimentary in size and shape to the template molecule. Additionally,

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the functionalities inside this cavity reinforce the selective retention of the target molecule on to the MIP. This synergy established between these two factors is the added value that MIPs have to offer over other sorbents used in SPE.

Fig. 1 illustrates the general procedure of obtaining the MIP. In the first step, the template molecule and the functional monomer are brought together to form a complex (1, 2). Afterwards, the cross-linking agent is added and polymerisation begins (3). The final step is to remove the template molecule used in the synthesis of the polymer (4), leaving behind cavities with the same shape, size and functionalities arranged in a special fashion to attract the target molecule.

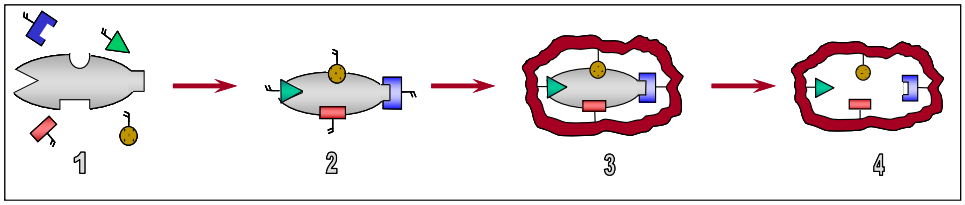

Fig. 1. General steps in the synthesis of a molecularly imprinted polymer.

The way to prove that the polymer produced is actually imprinted is by testing the affinity that the polymer synthesised has towards the template molecule and compare this affinity with a polymer obtained using the same synthetic protocol and monomer composition but without the template molecule. This polymer is known as a control polymer or non-imprinted polymer (NIP). Once the two polymers have been obtained, there are two general procedures for testing the affinity of the MIP towards its target molecule: one is by testing a parameter known as the Imprinting Factor (IF), which involves a chromatographic evaluation, and the other is by performing rebinding assays.

The IF value is a parameter derived from the different retentions that the template molecule used in the synthesis of the polymer has on the MIP and on the NIP. To obtain this parameter, part of the useful fraction of the MIP and NIP particles are packed in separate columns and these columns are used as stationary phases in a LC system. Afterwards, a solution spiked with the template molecule used during the polymerisation process (which is expected to have strong interaction with the imprinted polymer) is percolated through the MIP and NIP columns with another molecule that has no interaction at all with the polymer. This molecule is known as a void marker. The next step is to calculate the retention factor (*k'*) of the template molecule on the MIP and the NIP. This is calculated using the following equation: $k' = (t_{R,\ Template} - t_{R,\ Void\ Marker}) / t_{R,Template}$. In this way, a *k'* value is obtained for both MIP and NIP columns. Once each *k'* value is obtained, the IF is calculated as $IF = k'_{MIP} / k'_{NIP}$ [30,31]. The higher the IF value is, the higher the affinity of the MIP towards the

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target analyte. Another clue that suggests there will be a good imprinting effect left on the MIP is the broadness and the tailing of the peaks obtained when performing IF experiments. This is due to the different affinities of the binding sites to the template molecule, which arises during the synthesis of the MIP. Therefore, a high value of IF obtained for a MIP as well as a broad peak observed in the chromatographic valuation are clear evidences of the good imprinting outcome of the polymers.

Regarding rebinding assays, the affinity of the target molecule towards the MIP is obtained by comparing the different amounts of target molecule adsorbed on the MIP and on the NIP [32,33]. To do this, both the MIP and the NIP are left in solution with a known concentration of the target molecule in an organic solvent in order to favour the specific interaction of the MIP and the target molecule. Once equilibrium is reached, the different affinities of the polymers towards the target analyte are measured by comparing the different amounts of target molecule left in solution of the MIP and the NIP. In this way, higher recoveries are obtained in the MIP than in the NIP since the molecules are retained on the MIP through selective interactions.

The simplicity of the imprinting protocol has led to this technique being the technique of choice for synthesising highly selective sorbents in a very cost-effective way. The compounds normally used with this technique are rather rigid and small molecules. For large molecules, such as proteins, or molecules that can adopt many different conformations, such as oligomers, this approach has proven not to be as straightforward. The difficulty for the selective trapping of these molecules is inherent to the protocol of the polymerisation adopted. Because, in many cases, the synthesised polymer is very rigid and has a highly cross-linked matrix, the cavities generated within the MIP during the polymerisation process are a *frozen* print of the template molecule used. Therefore, the proper interaction in the cavities of the MIP for both large and highly-flexible molecules is difficult to establish. However, there are some examples in literature describing the synthesis of a MIP using a modified protocol with the same basic principle for imprinting proteins, as Shi *et al*. [34] described in the use of a polysaccharide scaffold on a solid support as a recognition element for imprinting proteins. Under these circumstances, the selective retention of those molecules remains a challenge to be overcome, unless a completely different approach on the synthesis of MIPs is adopted.

The main reason for the widespread use of MIPs arises from the wide range of commercially-available functional monomers which suit all of the different properties any given molecule might have. There are some reviews in literature illustrating this wide variety of functional monomers, as Zurutuza *et al*. [35] or Karin *et al*. [36] reported. However, the functional monomers mostly used thus far have been methacrylic acid, 2-(trifluoromethyl) acrylic acid and 4-vinylpyridine whose structures are shown in Fig. 2.

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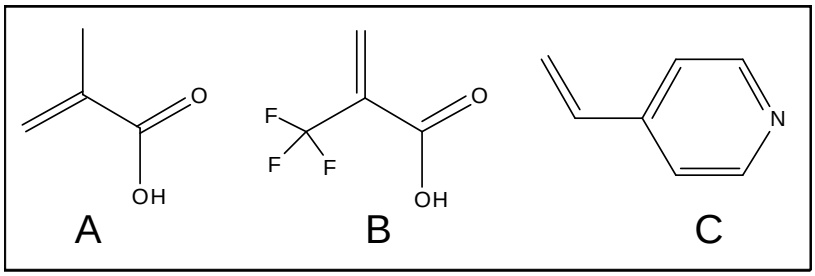

Fig. 2. Structures of the functional monomers mostly used in the synthesis of MIPs: (A) Methacrylic acid, (B) 2-(Trifluoromethyl) acrylic acid and (C) 4-Vinylpyridine.

In order to achieve the best interaction possible between the template molecule and the functional monomer and, in turn, to deliver the best recognition properties to the final MIP obtained, the functional monomers are sometimes specially designed for the target molecule. In this way, the functional monomer perfectly matches the functionalities that the template molecule has and a more efficient extraction of this molecule can be achieved. Sellergren *et al*. have extensively reported on the practice of this approach to obtain MIPs for extracting several analytes of interest from different matrices. Some examples of this group are the selective extraction of riboflavin [37] and the extraction of β-lactamic antibiotics [38] amongst others.

When designing the MIP, the ideal functional monomers are those with complimentary functionalities to those present in the template molecule. In this way, the best possible interaction between these molecules is obtained and a MIP is delivered with the desired properties.

During the synthesis of a MIP when a commercial functional monomer is used, an important decision has to be taken regarding the ratio of the functional monomer to the template molecule because, depending on this ratio, different properties of the final product can be obtained. On the one hand, when the functional monomer is present in much larger amounts than the template molecule, the MIP obtained may have many non-selective interactions. Because of this, the selectivity of the polymer may be hidden somehow by the great number of these interactions. On the other hand, when the amount of functional monomer to the template is not large enough and because the selectivity of the MIP arises from the proper self-assembly of the template molecule and the functional monomer prior to the polymerisation process, the selectivity of the MIP obtained may be jeopardised by a non-proper interaction of those two molecules.

Apart from the template molecule and the functional monomer, the third party involved in the synthesis of a MIP is the cross-linking agent. This molecule has, at least, two polymerisable double bonds in its structure. Therefore, as the polymeric chains grow, introduction of the cross-linking agent enables it to link to these growing

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chains, leading to a three-dimensional network that will be the final polymer. The role of this molecule is not as important as the functional monomer regarding selectivity issues but, depending on its nature and the application intended, accurate selection is required to avoid non-selective interaction of the MIP with other components present in the sample. Regarding the cross-linking agents, the most widely used so far having two polymerisable double bonds have been divinylbenzene (DVB) and ethylene-glicol dimethacrylate (EGDMA). As cross-linking agents incorporating three polymerisable double bonds in their structure, pentaerythritol triacrylate (PETRA) or trimethylolpropane trimethacrylate (TRIM) are some of the candidates reported, although the use of these cross-linking agents is not as common as those with two polymerisable double bonds. The structures of all these compounds are depicted in Fig. 3. Even though the ratio of cross-linking agent to template molecule is not as important as the ratio between the functional monomer and the template molecule, its concentration is also crucial so that the polymer has the intended mechanical properties. When the concentration of the cross-linking agent is too low, a polymeric gel-type formation is obtained instead of a macromolecular rigid structure because it does not achieve the minimum extent of cross-linking in the polymeric matrix.

In order to obtain a MIP with not only the selectivity but also the mechanical properties desired, all the molecules involved during the synthesis of the MIP must be in a suitable ratio for meeting the goal intended. The ratios most commonly used for obtaining MIPs range from 1:4:20 to 1:8:40 (for template:functional monomer:cross-linking agent). Using these particular ratios, a good balance between the selective and mechanical properties of the polymer is achieved.

With these ratios, the template molecule is both present in a concentration big enough to deliver a good imprint effect on the polymer and also low enough so that the final mechanical properties of the polymer are not modified.

During the synthesis of the MIP, to more or lesser extent, the use of a solvent is unavoidable. Its main function is to bring all the components involved in the polymerisation process together. Therefore, the polymerisation mixture becomes a homogeneous solution avoiding agglomerates or stratification in some points. In this way, the template and the functional monomer can establish proper interactions. Another function that solvent has is to bring porosity to the polymer. Porosity is essential for all of the sorbents used in SPE protocols. The more porosity a polymer has, the larger its surface area and so its capacity for enabling larger volumes of samples to be analysed is bigger. Since concentrations of the analytes of interest are rather low in most cases, larger volumes of samples make it easier to identify low concentration levels. However, in the case of MIPs, porosity of these polymers is more appealing for obtaining availability to the different actives sites fomed within the polymer rather than to allow large volume of sample to be analysed since the main use of MIPs thus far has been to perform strong clean-up of the sample.

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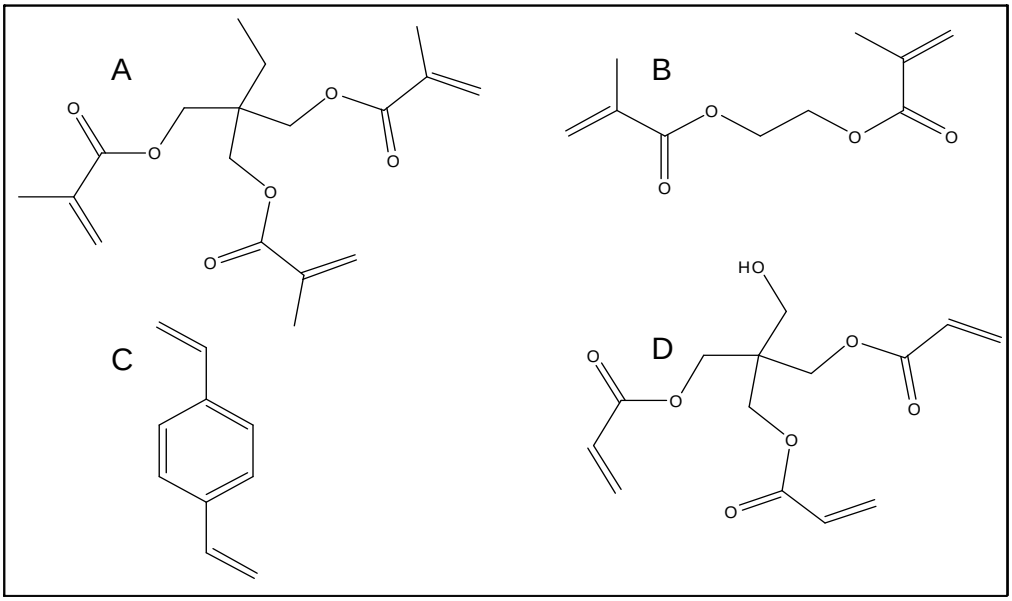

Fig. 3. Structures of the cross-linking agents most widely used in the synthesis of MIPs. (A) Trimethylolpropane trimethacrylate (TRIM); (B) Ethylene glycol dimethylacrylate (EGDMA); (C) *p*-Divinylbenzene (DVB) and (D) Pentaerythritol triacrylate (PETRA).

Another crucial parameter related to the solvent is its content in the polymerisation mixture. Cormack *et al.* [35] reported on the different polymeric outcomes that can be obtained depending upon the different percentages of the cross-linking agent present in solution. This study clearly demonstrates that the ratio of cross-linking agent and the volume of solvent is crucial for obtaining either gel-type materials or solid and macroporous polymers. In a highly diluted system or when the concentration of a cross-linking agent is too low, polymerisation reaction occurs but the product obtained is gel-type amorphous and, in the case of MIPs to be used in MISPE applications, this state is not normally desired.

In the case of MIPs for SPE applications, the polymer obtained must have strong rigidity and be a porous material and these conditions can only be reached when fine tuning the volume of porogen and the cross-linking ratio.

Selection of the solvent must also be done very carefully. As a rule, polar and protic solvents are preferably avoided. The solvents of choice are apolar with a low dielectric constant. The solvents most widely used that meet these features are acetonitrile and toluene, although dichloromethane [39,40] and chloroform [41,42] have also been widely used.

Because interactions of the template and the functional monomer are mainly achieved by hydrogen bonding and electrostatic interactions, apolar and aprotic

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solvents are preferred because they favour these kinds of interactions. Solvents that disrupt them are avoided.

However, there are some papers published in which polar and protic solvents were used as porogens and the MIP obtained using these solvents showed good recognition properties for the applications intended. For example, Surugiu *et al*. [43-45] described the synthesis of a MIP selective for Ibuprofen using a mixture of MeOH:water as porogen solvent and the MIP obtained showed good results on rebinding the target analyte. Our group [46] also has experience of using this kind of solvent mixture in the synthesis of a MIP and the further extraction of naphthalene sulfonates from water. More recently, Yan *et al*. [47] reported on the use of this solvent mixture for obtaining a MIP to extract enrofloxacine and ciprofloxacine from milk samples.

All the roles that the different components involved in the production of MIPs play as well as all the other considerations discussed so far must be taken into account when synthesising a MIP. There are several ways to produce MIP and following there is a description of the mostly widely-used polymerisation protocols thus far for synthesising them.

1.1.1 Traditional polymerisation

Since Sellergren [17] reported on the synthesis of the first MIP to be applied in SPE, Traditional Polymerisation has been the most widely-used protocol for obtaining imprinted materials as seen from the large number of publications reporting on its use for obtaining imprinted materials. The reason for such widespread use is the ease of the synthetic protocol, the low synthetic skills required for obtaining the MIP and the fact that this synthetic protocol can be performed in almost any laboratory.

In this kind of polymerisation, all the compounds involved in the process are mixed together in a perfectly dry vessel with a low volume of solvent and are left to polymerise.

Because of the low volume used in this approach and because most of the components involved are liquid in nature, this polymerisation is also known as *Bulk Polymerisation*. However, to our understanding, this nomenclature is not strictly correct since the term *Bulk Polymerisation* refers to a polymerisation in which strictly no solvent is used and the liquid media is generated because all the components involved in the polymerisation are liquid in nature. From this perspective and because this protocol uses a small volume of solvent, this polymerisation cannot be referred to as *Bulk Polymerisation*.

To achieve this polymerisation, it is desirable, but not mandatory, to add the compounds in the following order: template molecule, functional monomer, cross-

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linking agent, porogen and initiator agent in order to favour the interaction between the template molecule and the functional monomer. The volume of solvent generally used in this protocol is 4/3 of the sum of the masses of the functional monomer and the cross-linking agent involved in the polymerisation process. This will produce a macroporous polymer.

Once all the components are mixed together in the reaction vessel, the polymerisation mixture is thoroughly degassed with an inert gas (generally N_2) in order to remove all of the oxygen dissolved in this mixture. This is because oxygen can inhibit the polymerisation process. The degassing step is normally carried out in an ice bath in order to minimise the possible evaporation of any of the components present, especially those at the lowest concentrations.

Once the polymerisable mixture is completely degassed, the flask must be quickly and tightly closed so that the mixture is ready to polymerise.

The polymerisation process is generally carried out under either UV radiation or by thermal polymerisation at around 60 ºC. The first method is normally used when any of the compounds involved in the polymerisation process is thermally unstable or low temperatures are required for whatever reason [48,49]. Thermally-initiated polymerisation is the most commonly used protocol and is normally carried out at 60 ºC in an oil or water bath [50,51].

In any of the initiation processes described, the initiator molecule decomposes to produce two metastable radicals. Those radicals react under free radical polymerisation with any polymerisable double bond in solution. Although this reaction is pretty quick, the reaction is left to proceed for 24 or 48 hours at which point the vessel is removed, either from the UV radiation or from the oil bath.

Once the reaction is complete, the polymer is obtained in a monolithic form with the shape of the containing vessel and, in order to obtain the useful particles for the application intended, the monolith must be crushed, grounded and sieved. This process is not only very tedious and time-consuming but also the yield of the useful particles - *i.e*. the mass of the useful particles compared to the total mass of the polymer produced - is generally low. Fig. 4 shows the basic steps in a typically traditional polymerisation for obtaining MIPs.

The useful particles obtained under this protocol are irregular in size and shape because, once the reaction is complete, the polymer obtained is a monolith which has to be crushed, ground in a mortar and wet-sieved to obtain the useful particles. Due to the sieving process, the particles obtained under this protocol and which are used for MISPE application, usually range from 25 µm to 60 µm. This is because smaller particles would produce low flow rates and high back-pressures, whereas larger particles would not enable a proper mass transfer of the analyte on to the solid phase.

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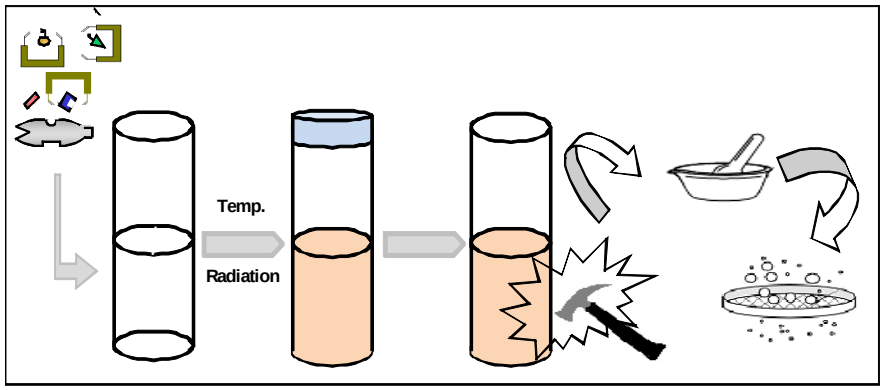

Fig. 4. Common steps for obtaining a MIP by Traditional Polymerisation.

A picture of how the final product obtained after processing the monolith under traditional polymerisation protocol looks like is shown in Fig. 5.

Despite the fact that this protocol is very straightforward to produce, there are some drawbacks that have to be taken into account when designing MIPs. Most of these drawbacks are due to the fact that the imprinted particles are obtained by crushing the monolith. Due to this process, these particles are irregular in size and shape. This makes it very difficult to properly pack the cartridges or columns where these particles are to be used. The irregular particles also make it difficult to perform an effective mass-transfer of the analyte to the stationary phase. Another important drawback to this kind of polymerisation is the fact that many active sites within the polymer are destroyed because of this process and, therefore, useful active sites already formed within the polymeric matrix are rejected. This issue is particularly important when the template used is scarce or expensive.

However, despite these drawbacks, this kind of polymerisation has been the most widely used process since MIPs became to be used as sorbents in SPE. Many new applications using MIPs are still being produced by this synthetic protocol, as seen from the great number of publications describing this protocol. As previously mentioned, this protocol has been used to synthesise the first MIP applied in MISPE applications in which pentamidine was extracted from urine samples [16]. Other examples are the extraction of cocaine metabolites from aqueous samples [52] or the extraction of ciprofloxacine from human urine samples [39].

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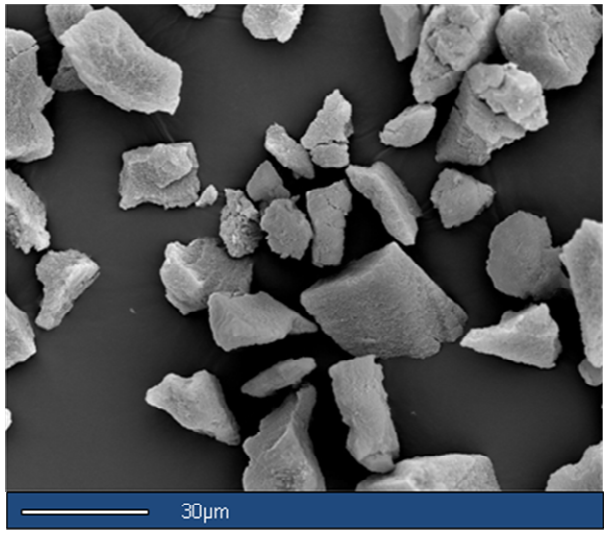

Fig. 5. Typical particles produced after processing the monolithic polymer obtained by Traditional Polymerisation.

1.1.2 Precipitation polymerisation

There have been many different synthetic approaches developed to overcome the irregular size and shape of the imprinted particles produced by Traditional Polymerisation. The most streamlined protocol is Precipitation Polymerisation, which produces spherical particles in a single preparative step within the normally used range of particle size for SPE applications. Fig. 6 shows the product obtained from this polymerisation protocol.

The discrete spherical particles are formed by their precipitation off the solution due to the cross-linking of the polymeric chains as they grow within a highly diluted system. However, there are some examples published which have used low concentrations of cross-linking agents, causing the obtainment of gel-type polymers. Although gel-type polymers have poor mechanical properties, good recognition for the target analyte can be obtained as Suede *et al.* reported on the extraction of dopamine and adrenergic compounds from urine [53], or as Hirayama *et al.* showed when obtaining a MIP for lysozyme from aqueous solutions [54].

Contrary to Traditional Polymerisation, this kind of polymerisation uses a process in which all the components involved in the synthesis of the polymer are highly diluted within a solvent (or a mixture of two solvents). Not only must this solvent be able to solubilise all the components involved in the polymerisation process at the temperature of polymerisation but also, as the polymeric chains grow, to be able to solubilise this polymer too, but only to certain extent. Once the polymer reaches a certain critical mass, this solvent is no longer able to hold the polymeric chain in solution, thus precipitating the particles off the solution.

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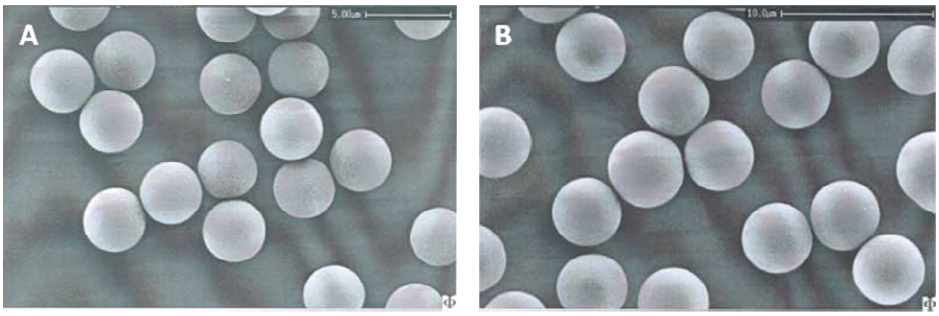

Fig. 6. Typical microspheres produced under PP protocol for an imprinted polymer using theophylline as a template molecule (A) and its control polymer (B) [55].

The temperature in which the solvent is able to hold the polymerisation process up to a certain critical mass - the polymer precipitates off the solution after this point - is known as *theta* (Θ) temperature. Solvents with this particularity for a given polymer are known as Θ solvents. Therefore, the selection of a proper Θ solvent is essential to obtain discrete particles in this process. This is because the desired product cannot be obtained if either the solvent solubilises the polymer at the polymerisation temperature selected or does not solubilise the oligomers formed in the first steps of the polymerisation process.

Sometimes, to tune the particle size, a mixture of two solvents is used. In these cases, the solvents are known as *good* and *bad* solvents. These adjectives refer to their ability to solubilise the growing chains during the polymerisation process. The *good* solvent allows the formation of polymeric chains and, depending upon the concentration of the *bad* solvent, these polymeric chains are precipitated off the solution at different critical masses, therefore delivering a different particle size. Even if just one solvent or a mixture of two is used in the polymerisation process, these solvents must also act as a porogen to make the formed particles porous.

The most widely used solvents to synthesise imprinted materials under this approach have been ACN and toluene, or a mixture of both solvents in which toluene is the *good* solvent and ACN is the *bad* solvent. In this case, toluene brings porosity to the polymer and keeps the polymeric chains growing in the solution from collapsing. On the other hand, ACN is responsible for precipitating the polymeric chains once they reach a certain critical mass and avoids the gel-formation of the polymer. However, this solvent system is not the only one that can be adopted for synthesising MIPs since, as Horváth *et al*. [56] recently reported, MIPs by precipitation polymerisation can also be obtained using oil as a solvent. In this case, the use of oil allowed the authors to use a higher monomer ratio during the polymerisation process. The MIP thus obtained performed in a very similar way to a MIP obtained using organic solvents. However, the control polymer obtained using oil as solvent showed similar results to the imprinted one.

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Another important factor to take into account when synthesising MIPs under this approach is the concentration of initial components involved in the polymerisation process. As Sherrington reported [57], a different polymeric product can be obtained depending upon the concentration of these different components. The typical ratio of monomer weight in solution for this kind of polymerisation is 3-4% (w/v) to the total volume of solvent. Lower or higher percentages may lead to gel formation.

An excellent discussion of all the parameters affecting the polymerisation process was reported by Wang *et al*. [58] in which the authors fully detailed the influence of each parameter involved in the polymerisation process. The authors described the importance of the functional monomer to match the Θ temperature of the solvent used in the polymerisation for obtaining the particles desired. Moreover, they also described the effect that the use of several solvents had on the final product and also reported on the best mixture and composition of solvents to be used in this protocol - this turned out to be a mixture of ACN:toluene 75:25. Another important piece of data reported in this paper was the concentration of functional monomer and the initiator used. This concentration must be balanced in order to obtain monodisperse spherical particles in good yields. Otherwise, the particle size, particle size distribution and the polymerisation yield would be dramatically affected. These authors also optimised the parameters concerning the method of mixing together all of the compounds involved in the polymerisation process to obtain the desired particles. They compared the agitation produced by a rotavapor system to that produced by a roller incubator and concluded that the roller incubator delivered better particles in terms of polymerisation yields and reproducibility of the particles obtained.

The proposed mechanism for the formation of the particles, as Downey *et al*. described [59], consists of two basic steps. In the first step, an aggregation of the oligomers in solution occurs and thus becomes the particle nuclei. The second step involves soluble oligomers being captured by the growing particle to become the final particle. This capture mechanism is more likely to be driven by the entropic energy of adding free radicals in solution onto the growing particles rather than an enthalpic mechanism caused by the desolvation of the free oligomers from the solution.

An important drawback to this kind of polymerisation is the fact that not so many solvent systems can be used for this synthetic protocol because their properties do not suit the requirements previously mentioned for the solvents used. Another important drawback is that, due to the huge effect that the presence of a template molecule might have during the synthesis of the polymer, the presence of such a molecule can even prevent the polymerisation process itself. The effect that a given molecule induces in a polymerisation system is well known and scientists working in polymer science have made use of and taken advantage of this effect. This effect is known as template directed synthesis [60,61]. This effect is attributed to the different reactivity induced by the presence of a template molecule on the polymerisable double bonds. This molecule might align and affect the reactivity of the polymerisable double bonds involved in a polymerisation process so the final product obtained

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might dramatically differ from the same product obtained without the presence of such a template molecule.

Under these circumstances, it is not difficult to understand that the presence of the template molecule during the synthesis of a MIP might play a crucial role in obtaining very different properties in the MIP or the NIP. For example, Sambe *et al*. [62] reported a MIP obtained under this kind of polymerisation by using nicotine as the template molecule. In their case, the polymerisation yield for the control polymer was twice the yield for the MIP, whereas the particle size for both polymers was almost the same. Another interesting example was reported by Wang *et al*. [55] after synthesising a MIP using theophylline as the template molecule. In this case, good polymerisation yields and a similar particle size were obtained for both the control and MIP polymers.

These results illustrate the different changes that can be observed in imprinted particles and that the particles obtained can still be useful for their intended applications.

Over the last few years, this kind of polymerisation has been used increasingly when synthesising MIPs because spherical particles of suitable size that are ready to be used in MISPE applications can be obtained in a single preparative step and in polymerisation yields ranging from moderate to good. For example, Cacho *et al*. [63] reported on the use of this polymerisation protocol for obtaining a MIP selective for triazines and their detection in soils and vegetable samples. Turiel *et al*. [64] also obtained a MIP using this protocol for detecting fluoroquinolones in soil samples.

1.1.3 Swelling polymerisation

This is another kind of polymerisation protocol aimed at overcoming the problems derived from Traditional Polymerisation. Its aim is also to deliver monodisperse spherical particles within the range of particle sizes normally used in sorbents for SPE.

In this polymerisation and in order to obtain the final spherical particles desired, a suitable organic solvent is added to a suspension of preformed spherical particles to swell their initial size. This step can either be performed once, and then this protocol is known as *Swelling Polymerisation,* or several times, thus becoming *Multi-Step Swelling Polymerisation* [65].

In any case, the initial particles are suspended in water and an activating agent and swelling agent is added. In this way, the initial particles of around 1 µm in diameter swell to a certain size and, if necessary, another swelling step to increase the particle size further can be performed. Once the particles are the desired size, all the components involved in the polymerisation process are added to the solution containing the swollen particles and polymerisation is induced to obtain the desired

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MIP. A schematic representation of this protocol is shown in Fig. 7. In this figure, initial polystyrene particles of 1 µm in diameter are suspended in water. Afterwards, an activating agent (such as dibutyl phthalate) as well as a stabilising agent (such as sodium dodecyl sulfate) are added. Once both agents have been incorporated with the seed particles and, if the particles obtained do not have the appropriate size for the application intended, a second swelling step can be performed. This second step is normally carried out using a dispersion of a suitable organic solvent and a dispersion stabiliser agent (such as polyvinyl alcohol) in water. After the second, a third swelling step is performed using the typical components involved in a polymerisation process for obtaining imprinted polymers.

The final size of the particles then obtained is in the low micrometer range.

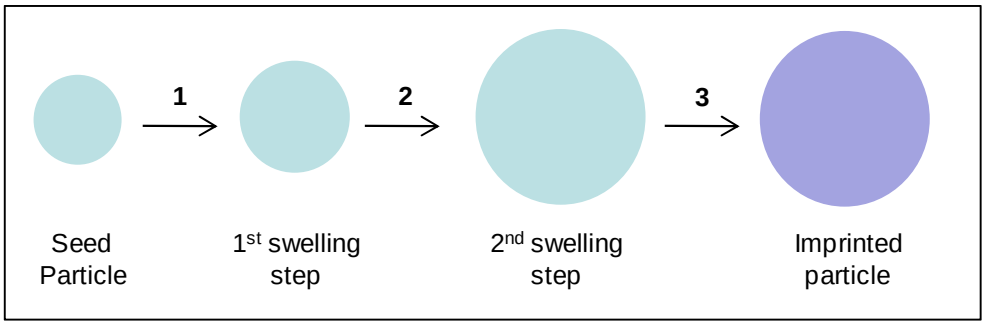

Fig. 7. Basic steps for obtaining imprinted particles by Multi Step Swelling Polymerisation.

The product thus obtained has narrow dispersity and a suitable size to be used as a sorbent in MISPE protocols.

The main drawback of this approach is that the water is generally used as a dispersing agent during the successive swelling steps in this polymerisation protocol. Since self-assembly of the template molecule and the functional monomer is normally achieved through a hydrogen bond or electrostatic interactions - and water is a molecule that is liable to establish this kind of interaction - the presence of water during the polymerisation process is preferably avoided. Therefore, the possible effect that this molecule could have over the proper interaction between the template molecule and the functional monomer would be diminished.

Despite the presence of water, there are many papers in bibliography describing the use of this kind of polymerisation to obtain several MIPs. The group of Haginaka is one of the most active groups, preparing imprinted polymers using either Swelling or Multi-Step Swelling Polymerisation protocols over the last few years. This author pioneered the first approach to the synthesis of a MIP using this polymerisation technique. In that case, the authors used naproxen as the template molecule [65]

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and the polymer obtained showed enhanced selectivity towards the template molecule used during its synthesis than to other closely-related structures when the polymers were tested as stationary phases and using aqueous-based mobile phases.

Apart from these results, the group of Haginaka and has developed many different imprinted polymers using this methodology to selectively extract different analytes of interest such as barbiturates [66], triazine herbicides [67] or bisphenol-A [68], amongst others from water-based matrices such as river water.

Although this process has not been as well accepted as Traditional or Precipitation Polymerisation, there are some groups that have also used this protocol when synthesising their own MIPs. For examples, Fu *et al*. [69] developed a MIP under this protocol with nimodipine as the template molecule for its detection in plasma samples. Li *et al*. [70] also developed a MIP using this polymeric approach and used ephedrine as the template molecule for its enantiomeric separation.

1.1.4 Suspension polymerisation

Suspension Polymerisation is another synthetic protocol designed to deliver spherical particles with an appropriate size and shape to be used as the stationary phase in columns for liquid chromatography or as sorbents in SPE protocols.

Unlike multi-step swelling polymerisation but similar to precipitation polymerisation, this protocol creates the desired particles in a single preparative step.

This case is another example of a protocol that forms discrete particles in a highly-diluted system. However, unlike the precipitation polymerisation process, the solvent used in Suspension Polymerisation is not intended to hold the growing polymeric chains. Its function is, as in multistep swelling polymerisation, to be the dispersing media for the proper formation of the polymeric particles.

The first step in the general procedure for this polymerisation is to mix thoroughly together the dispersing solvent with a suitable stabilising agent. Afterwards, an immiscible solution to the dispersing agent, containing all the components involved in the polymerisation process (template molecule, functional monomer, cross-linking agent, porogen and initiator), is added carefully during vigorous stirring. The combination of high speed stirring and the immiscibility between the dispersing phase and the porogen leads to the formation of discrete droplets in the micron size range.

Once the droplets of the polymerisation mixture have formed within the dispersing phase, the polymerisation reaction is induced within any discrete droplet. In this way every droplet acts as a unique reactor. The typical size for the particles obtained under this procedure range from 5 µm to 100 µm.

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The most widely used dispersing agent in this kind of polymerisation process is water [32,71]. However, there are other cases in which a different solvent has been used. A particularly interesting case of synthesis of a MIP using this approach was reported by Wang *et al*. [72] in which the authors used silicon oil as a dispersing agent. In this case the template molecule was 2,4-dichlorophenoxyacetic acid and the MIP subsequently obtained showed enhanced selectivity towards its template molecule compared to the control polymer.

Another approach for obtaining MIPs under this synthetic protocol that avoids the use of water as a dispersing agent is to use fluorocarbons as dispersing agents. This solution was first proposed by Mosback *et al*. [73] and the authors demonstrated that the use of perfluorocarbons did not interfere with the interactions between the functional monomers and the template molecule during the polymerisation process and that the MIP obtained showed higher affinity towards its template molecule than the control polymer. These authors also used this protocol in further experiments [74] but, due to the hazard the use of these solvents represents, especially to the environment, their use is preferably avoided.

Mayes *et al*. [73] also reported on a series of MIPs obtained using perfluorocarbons as dispersing agents. The authors concluded that the use of perfluorocarbons could help in obtaining discrete spherical particles of a suitable size for MISPE applications and that perfluorocarbons are easy to handle and can even be recovered after the polymerisation process.

Despite the advantages that perfluorocarbons as dispersing agents might bring when obtaining MIPs by suspension polymerisation, the MIPs that have appeared over the last few years still make use of water as a dispersing agent. This is the case with Qu *et al*. [75] who reported a MIP for extracting several antibiotics from milk. Bunte *et al*. [76] also reported on a MIP obtained under this polymerisation technique for detecting 2,4,6-trinitrotoluene from vapours containing this explosive and a high affinity of the MIP was demonstrated.

The main drawback of using water as the dispersing agent is that when either hydrophilic templates, functional monomers or cross-linking agents are used in the polymerisation process, partition of these compounds in the two different solvents is unavoidable and therefore diminishes the number of actives sites which would be formed in the polymeric matrix.

1.1.5 Grafting procedures

The use of this polymerisation protocol in MISPE applications, as well as in all the previously described polymerisation methods, aims to deliver spherical particles within the appropriate size for MISPE applications and thus overcoming the drawbacks of traditional polymerisation.

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There are two different approaches to obtaining MIPs under this protocol: *Grafting To* and *Grafting From* protocols. In any of these two protocols, a different component involved in the polymerisation process is grafted on to the surface of the silica particle. Once this component is attached to the silica particles, all of the rest of the components involved in the polymerisation process are added to the solution and the polymerisation process is induced.

At this point it is worth noting that the term *Grafting To* when obtaining MIPs and its further application in MISPE protocols is not strictly correct. This is because what *Grafting To* means in the field of polymer science is the attachment of a whole preformed polymer on to a surface. However, in imprinting technology, *Grafting To* is a well known synthetic protocol for delivering imprinted polymers, although its use in obtaining MIPs and applying them under MISPE techniques has not been described.

Before explaining what is the most commonly understood *Grafting To* definition, it is worth pointing out that a different definition was proposed by Ulbricht [77]. According to this author, what *Grafting To* means is and, as stated previously, the direct attachment of a preformed polymer on to a surface and, so far, there is no reference in any literature to this technique for obtaining MIPs to be used in MISPE applications. However, and also according to this author, another definition for *Grafting To* is the coating of a surface with reactive monomers involved in a polymerisation process in order to improve the adhesion and formation of a polymer on a given surface. The second definition is the most widely understood in terms of the imprinting process.

In any case, with "*Grafting To*" protocols for obtaining the desired MIPs, the component grafted on to the surface of the silica particles is the functional monomer. Once this compound is attached to the silica, an exact volume of the solution containing all the compounds involved in the polymerisation process is carefully added to the particles. This volume corresponds to the total volume of the silica particles and, once all the silica particles are filled with the prepolymerisation mixture, the reaction is induced.

In the mid-1980s, Mosbach *et al*. [78,79] developed imprinted polymers using Rhodanil blue and Safranine O, respectively, as template molecules and the authors observed preferential uptake for the template molecules used in all of the MIPs synthesised when these sorbents were applied as stationary phases in liquid chromatography.

However there is a big drawback to this technique in that there is no control over the thickness of the polymeric layer formed. Therefore, once the MIP is obtained, the rebinding and release of the target molecule to and from the polymer is dependent upon the polymeric thickness.

Due to this inconvenience, this approach has not been adopted for obtaining MIPs and there are no MISPE applications using sorbents synthesised under this protocol.

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Regarding *Grafting From*, this approach aims to overcome the drawback of the *Grafting To* approach. In this case, the compound attached to the surface of the silica particles is the initiator agent.

In this approach, the polymerisation reaction proceeds from the silica particles outwards, so there is more control over the thickness of the polymeric layer formed and, therefore, the rebinding and release process is improved.

An example of this approach was reported by Sellergren *et al.* [80] in which the authors described the use of this technique for the synthesis of a MIP using L-phenylalanine as the template molecule.

Despite the advantages this protocol has over the *Grafting To* protocol, its main drawback is that, once the polymerisation reaction is started, a radical is left in solution that, in turn, can also start a polymerisation process. When this happens, the polymer formed from this reaction tends to form aggregates or to merge different silica particles.

There are several ways to overcome this drawback. One is to use a kind of initiator agent known as initiator-chain transfer (iniferter) agents. These agents are asymmetric initiating agents which have two parts. Once the reaction has started, the first part of the initiator agent proceeds the polymerisation reaction whereas the second part is able to stabilise radicals. It then joins to the end of the propagation chain, thus terminating the polymerisation process.

This approach was adopted by Baggiani *et al.* [81] for the synthesis of a MIP using the fungicide pyrimethanil and was successfully applied in a MISPE protocol for detecting this compound in wine samples.

Another approach to address the problem derived from directly grafting the initiator agent to the surface of the silica particles is by using chain transfer agents. These agents enable better control of the kinetics of the polymerisation process, a linear conversion over polymerisation time and the production of a more homogeneous polymeric layer. This approach was used by Sellergren *et al*. [82] to obtain a MIP selective for L-phenylalanine. The authors successfully applied the polymer obtained as stationary phase for LC separation of the two enantiomers of the template used.

The main advantage that grafting techniques offer over other polymerisation protocols is that the particle size of the polymer obtained is known in advance by knowing the size, shape and porosity of the original silica particles and an accurate idea of these properties in the final product can easily be obtained. In this polymerisation process, the silica particles are completely but not excessively filled with the polymerisation mixture and then polymerisation is induced. Once polymerisation is complete, the silica particles are etched away by using either HF or NH_4HF_2 and, finally, the polymeric product obtained is the specular image of the original silica particles.

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1.2 Approaches for the synthesis of MIPs

As mentioned previously, the high selectivity of MIPs arises from the stronger retention of the target molecule on the sorbent, rather than the rest of the molecules present in the sample. This retention is directly related to the proper interaction of the MIP and the target analyte that, in turn, is related to the interaction established between the functional monomer and the template molecule used during the synthesis of the polymer. Under these circumstances, the better the interaction between the target molecule and the MIP, the higher the affinity of the target molecule on to the MIP.

Therefore, the key factor for a successful imprinting protocol is a proper interaction between the template molecule and the functional monomer prior to and during the polymerisation process. This is because the proper rebinding of the target analyte to the MIP will depend on this interaction.

Before the polymerisation process, interactions between the template molecule and the functional monomer can only be through either covalent or non-covalent interactions. Once the polymer is obtained, retention of the target molecule to the MIP can also be achieved through covalent or non-covalent interactions. Therefore MIPs can be classified depending upon the interaction established between the functional monomer and the template molecule before the polymerisation process and the interaction of the target molecule and the MIP once the polymer is obtained.

MIPs are therefore classified as being obtained using the non-covalent, covalent or the semi-covalent approach, which will be discussed below.

The *Non-Covalent* approach has been the most widely used approach in the synthesis of MIPs. There are three main reasons for this success: the first is because it is the most streamlined synthetic approach developed so far; the second is because there is a full range of commercially-available functional monomers [35] which cope with all the possible functionalities that a given target molecule might have and, thirdly, is the fact that almost any molecule can potentially be used as a template in the imprinting process.

In this approach, a suitable functional monomer is left in solution with the template molecule and self-assembly occurs by non-covalent interactions. Then, the rest of components of polymerisation are added and polymerisation is induced. Once the polymer is obtained, rebinding of the target molecule is also achieved through non-covalent interactions. Fig. 8 shows a schematic representation of the synthesis of a MIP using this approach.

The non-covalent interactions involved in this approach can either be hydrogen bonding, ionic interactions, metal-ligand coordination, disulphur interactions or electrostatic interactions.

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In this approach, an excess of functional monomer is normally used to displace the equilibrium to the complex formation and to ensure all the functionalities of the template molecule are fully copied. This excess of functional monomer usually ranges from 4:1 to 10:1 in respect to the template molecule. However, this excess of functional monomer also implies the introduction of a great number of functional monomers randomly incorporated into the matrix of the polymer which, once formed, will bring a large number of non-specific interactions.

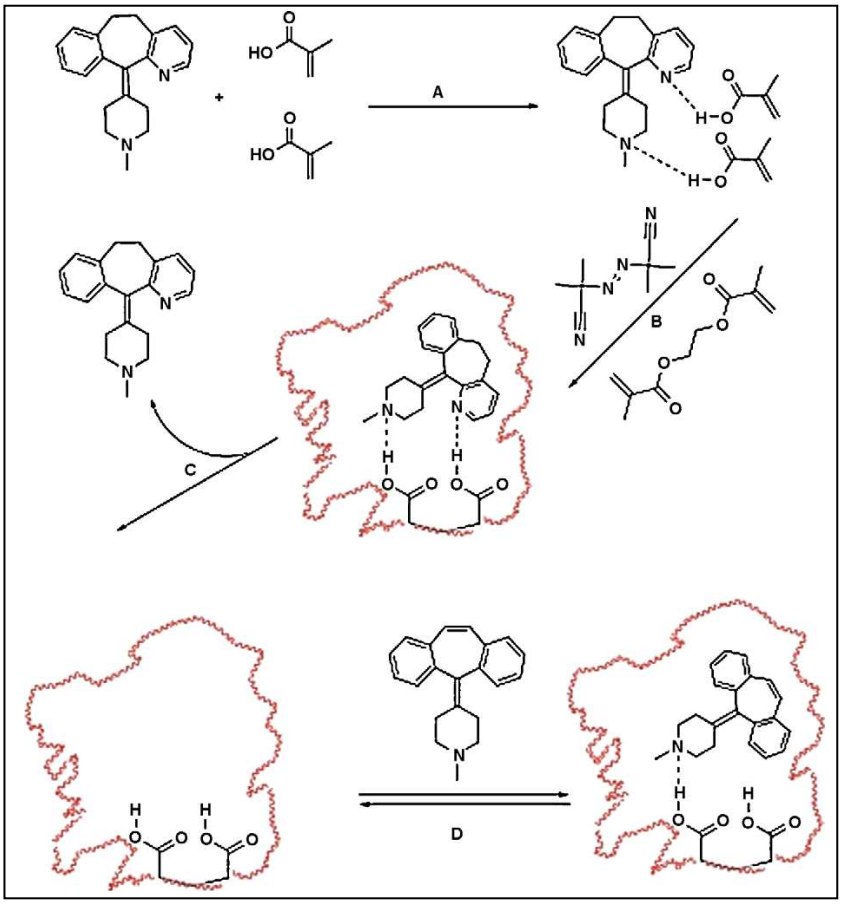

Fig. 8. A schematic representation of the synthesis of a MIP. Step A: mixture of the template molecule and the functional monomer (MAA in this case). Step B: addition of the cross-linking agent and initiator (EGDMA and AIBN, respectively). Step C: removal of the template molecule in the synthesis. Step D: rebinding of the target analyte within the cavity produced in the polymer [83].

The main drawback of this approach is the fact that, since the interactions of template molecule and functional monomer during the polymerisation process are

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non-covalent and polymerisation is an exothermic process, the complex formed can be slightly deformed during the polymerisation process. Once the MIP is obtained, the possible deformation of the complex template molecule-functional monomer causes heterogeneity amongst the binding sites. Another common drawback for this synthetic approach is the fact that, once the polymer is obtained, if there is any template molecule still trapped within the matrix, it may elute during the normal use of the polymer as a sorbent, masking the results obtained. This process is known as bleeding of the cartridge. The solution adopted to overcome the bleeding problem is to incorporate an analogue of the target molecule when aiming to detect low concentrations of the analyte of interest [52,84,85].

This approach is the most widely used in molecularly imprinting technology since any molecule can be imprinted and the availability of functional monomers makes this approach very suitable for obtaining imprinted materials.

When combining the availability for obtaining functional monomers for any given functionality a molecule might have and the feasibility of obtaining polymeric materials by traditional polymerisation, it is not difficult to understand why the use of MIPs as selective sorbents has rocketed over the last few years and why the use of targeted sorbents is still being widely exploited in many different fields and for many different applications.

The main characteristic of the *Covalent Approach* is the fact that interaction between the template molecule and the functional monomer before the polymerisation process is covalent and the interaction of the target molecule and the functional monomer once the polymer is obtained is also through a covalent bond.

MIPs obtained under the covalent approach have some interesting advantages over MIPs obtained under the non-covalent approach because the template molecule is covalently attached to the functional monomer. This means that a non-excessive amount of functional monomer is required in the synthetic process, diminishing the non-selective interactions during the rebinding process. Homogeneity amongst all the binding sites and well-defined interaction sites are also achieved because they all came from the cleavage of the template molecule. Fig. 9 depicts the basic steps involved in producing a MIP under the covalent approach, as well as the rebinding of the target analyte on to the MIP.

Despite the fact that this was the first approach used in imprinting processes and the theoretical advantages that this approach brings over other existing ones, this protocol is not routinely used when designing MIPs for SPE. The main reasons for this are that functional monomers cannot be attached to all the analytes of interest and also because of the low kinetics involved in both the rebinding of the target analyte onto the MIP and its further removal due to the covalent bond involved in these processes. Another important drawback is the lack of functional monomers that

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are able to establish a covalent bond with the target molecule under the mild conditions required in MISPE protocols.

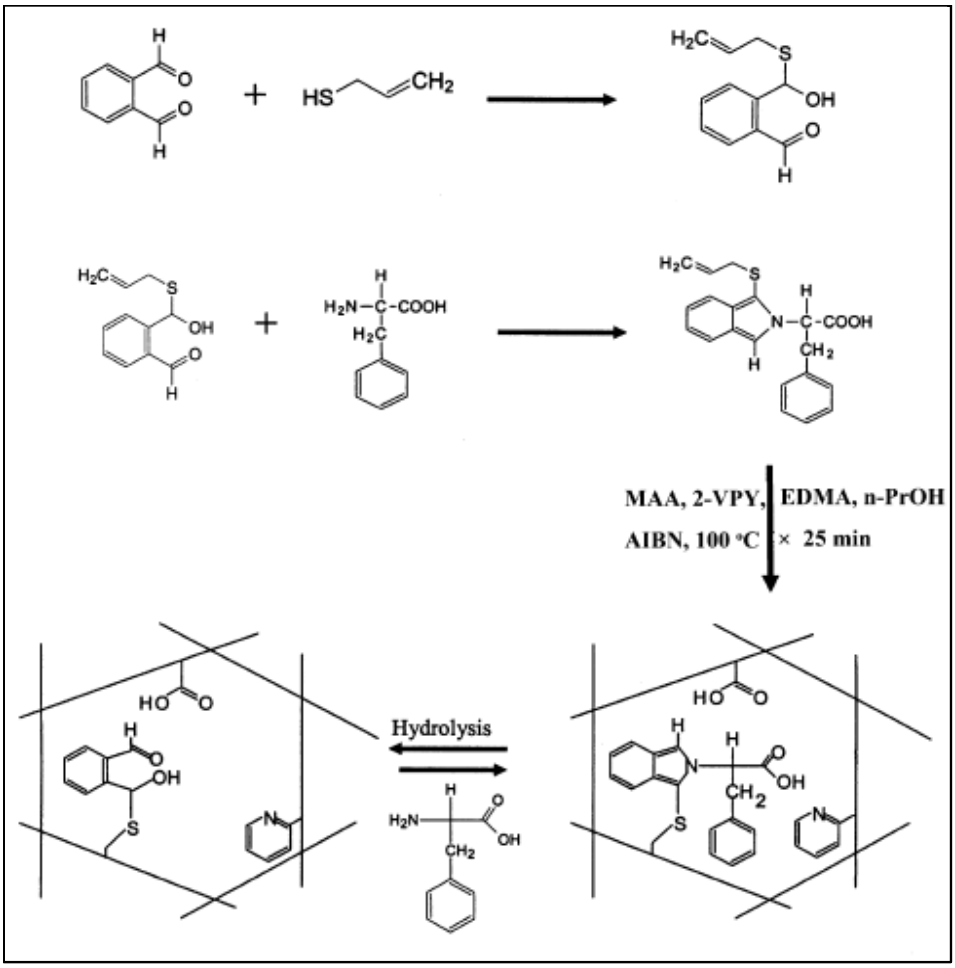

Fig. 9. A schematic representation of the synthesis and rebinding of the target molecule under the covalent protocol [86].

In this case the authors brought *o*-phthalaldehyde to react with allyl mercaptan to obtain the functional monomer required. Once the functional monomer was obtained, it was brought to react with L-phenylalanine, which was the target analyte, and the polymerisation process was induced using this “supra-structure” of the template-molecule with the functional monomer. In addition, the authors used other functional monomers not covalently bonded to the template used. This was done to further

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enhance the affinity of the active sites generated within the MIP to the target molecule.

As stated previously, the covalent approach has not been used to synthesise MIPs for SPE. However, if they were, it is expected that these MIPs would also suffer from the same bleeding problem as the MIPs obtained by the non-covalent approach. This is because the interaction between the template molecule and the functional monomer before the synthesis of the MIP and the interaction of the target molecule and the MIP during the rebinding of the target molecule is the same in both approaches. Therefore, to avoid the bleeding problem with the covalent approach, the solution adopted would probably be very similar to the one employed when using the non-covalent approach, i.e. using template analogues.

In the *Semicovalent Approach*, prior to the polymerisation process, the template is covalently attached to the functional monomer and, once the polymer is obtained, rebinding of the target molecule is through non-covalent interactions. Therefore, this approach combines advantages from both the covalent and non-covalent approaches.

The advantages of the covalent approach are that it tries to keep homogeneity amongst the binding sites, has uniformity on the interaction sites and does not use an excess of functional monomer (therefore diminishing non-specific interactions). The main advantage of non-covalent interactions is the fast kinetic uptake and release of the target analyte.

Contrary to the previously reported approaches, no bleeding is expected with this particular approach. This is because if there were a template molecule still remaining within the matrix, it would still be covalently bonded to the polymer and the mild conditions used to disrupt non-covalent interactions are not expected to be able to cause cleaveage of the covalent bond of the remaining template.

However, the semi-covalent approach suffers from the inconvenience that not so many template molecules can be attached to a functional monomer and that it is not as straight forward as the non-covalent approach.

Because of the interaction between the functional monomer and the template molecule, there is normally a single interaction point within the cavity using this approach. For this reason, retention of the target analyte on the polymer obtained under this approach is sometimes lower than for polymers obtained under different protocols.

Another drawback of the semicovalent approach is that, once the template molecule is removed from the MIP, the space generated within the cavity might not be large enough to properly accommodate the target analyte. This situation arises when the template molecule is directly attached to the functional monomer. In this case, to remove the template molecule, a covalent bond must be cleaved and, since

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rebinding of the target analyte to the MIP is through non-covalent interactions, these interactions need more distance to be established. Therefore, the cavity generated by the template molecule covalently bonded to the functional monomer during the synthesis of the MIP might not be able to properly accommodate a hydrogen bond interaction between the target molecule and the functional monomer.

The best way to overcome this drawback is by introducing a sacrificial spacer. A sacrificial spacer is a labile functional group (often a carbonate) which links the functional monomer to the template molecule and, once hydrolysed, generates an extra space within the cavity for optimal positioning of the target molecule in the imprinted binding site during the rebinding process.

This approach has been adopted by several authors when synthesising MIPs. For instance, Whitcombe *et al.* [87] adopted this approach for synthesising a MIP using cholesterol as the template molecule. This was attached to the functional monomer by a carbonate which acted as a sacrificial spacer. Another example was reported by He *et al.* [88] in which the authors used this approach when synthesising a MIP selective for testosterone. A schematic representation of the semicovalent approach using the sacrificial spacer solution is depicted in Fig. 10.

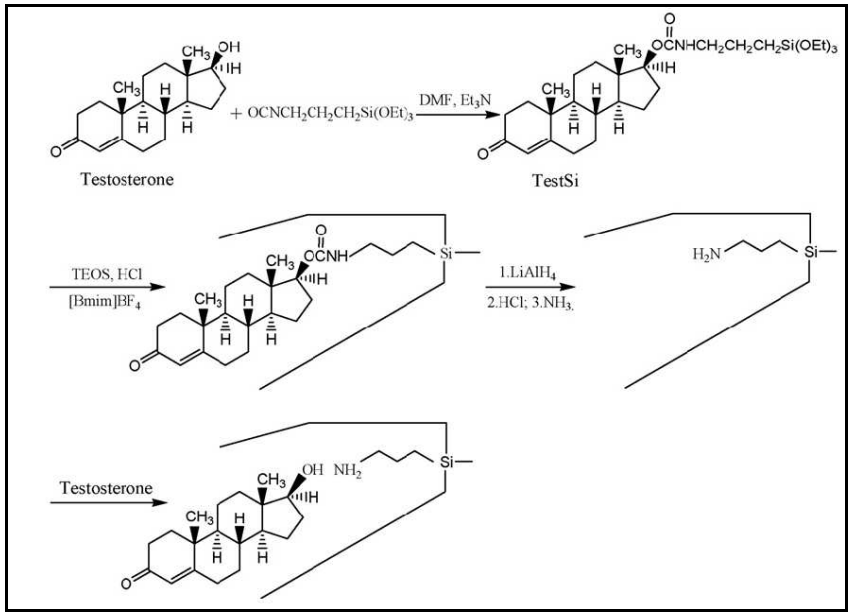

Fig. 10. A representation of the basic steps involved in the synthesis of a MIP under the semicovalent approach incorporating a sacrificial spacer [88].

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As can be seen in Fig. 10, the first step in the semicovalent approach is to attach the functional monomer to the template molecule (testosterone). However, since the authors incorporated a sacrificial spacer, they used 3-(triethoxysilyl)propyl isocyanate as a functional monomer and brought it to react with testosterone to obtain the functional monomer-template complex (TestSi), which incorporated a labile structure. Afterwards, the authors added the rest of the polymerisation compounds and induced the polymerisation process. Once the polymer was obtained, the template molecule was cleaved from the polymer together with the sacrificial spacer. Once the hydrolysis of the template molecule was completed, the target analyte could be rebinded to the MIP by non-covalent interaction and there was an extra space within the cavity that was derived from the cleavage of the sacrificial spacer used in the synthesis of the MIP.

Even though it has been used to synthesise MIPs for MISPE applications, the semi-covalent approach has not been as widely used as the non-covalent approach.

Over the last few years, the semi-covalent approach has been applied successfully for the extraction of the endocrine disruptor bisphenol-A in water and milk samples [51], flavanoid extraction from Ginko biloba [89] and the clean-up of triazines in soil and vegetable samples [63].

1.3 MIPs for Solid-Phase Extraction

The aim of the use of MIPs as sorbents in SPE is to achieve highly selective extractions of the target analyte. This is despite the fact that, as mentioned in the first part of this introduction chapter, immunosobents (IS) are probably the best candidates to perform such selective extractions or that even using mixed-mode sorbents a certain degree of selectivity can also be achieved in the extraction process.

A good evidence for this is that in the case of IS, due to both the difficulties in obtaining these sorbents and the srict conditions required for their use, IS are not so used to perform selective extractions. Regarding mixed-mode sorbents, despite these sorbents can retain compounds with similar polar functionalities, these sorbents do not allow to perform as selective extractions as MIP. An interesting comparison of a hydrophilic-lipophilic sorbent, a mixed-mode sorbent and a MIP was performed by González-Mariño *et al*. [90] when extracting amphetamine compounds from wastewater samples. In this study, the authors demonstrated that the best results were obtained when using a MIP as a sorbent because neither the standard sorbent nor the mixed-mode sorbent could reach the detection limits achieved when using the MIP due to the high ionic suppression arising from the matrix. The cleaner chromatogram obtained with a MIP reduces the ionic suppression when highly sensitive detection systems, such as MS, are used. This reduction in ionic suppression decreases the limits of detection when other SPE sorbents are used.

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Moreover, when comparing the hydrophilic-balanced sorbent with the mixed-mode one, recoveries for the former were poorer than for the latter. This indicates that a stronger discrimination for the analytes of interest obtained when using the mixed-mode sorbent is more effective than when using a hydrophilic-balanced one.

MISPE is a particular case of SPE so, essentially, in both cases, the same basic steps are performed. However, to make the most of all the advantages that MIPs offer over other kinds of SPE sorbents, a careful selection of the conditions of each step involved in the extraction protocol is required.

The first step is the conditioning of the cartridge. This step involves the percolation of a suitable solvent in order to bring the MIP to the optimal conditions for the further rebinding of the target analyte on to it.

The second step is the percolation of the sample. In this case, optimisation of the solvent used in this step is crucial since, depending on the nature of this solvent, the interactions established between the target analyte and the sorbent might be disrupted, leading to a null retention of the target analyte. This null recovery is explained because the interactions involved in the selective retention of the target analyte on to the MIP are mainly through hydrogen bonding or ionic interaction and these interactions can easily be disrupted, especially when using an organic solvent in the loading step.

The third stage is the clean-up step. In most cases, this is the most important step in the MISPE protocol. This is especially true with cases in which the MISPE protocol is performed directly on the sample of interest, as for water samples because, in these samples, the success of the whole protocol depends on this step since when loading the cartridge with an organic solvent sometimes this step is avoided. The goal in this step is to remove all of the compounds retained by non-selective interactions on the MIP while leaving the target analyte in place. This goal is achieved by carefully selecting a suitable solvent to disrupt all the non-selective interactions retained on the MIP while not disrupting the selective ones established between the target analyte and the MIP [52,81]. Ideally, once the MIP is properly conditioned and the sample percolated under optimal conditions, a suitable solvent is percolated through and the only compound retained on the MIP is the target analyte. The high selectivity of the MIP enables to extract the analyte of interest free from most of the compounds also present in the matrix, even when it is present in highly complex matrices. There are some examples where the very effective clean up performed on the sample has enabled a direct injection to the detection system of the analyte retained on the MIP with no need of any separation technique. To this end, our group [91] reported on the selective extraction of ciprofloxacine from urine samples and, after performing the clean-up step, quantification of this analyte was directly determined by mass-spectrometry, thus lowering the limits of detection and shortening the time of analysis.

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The last stage in a MISPE protocol is the elution step. The aim of this step is to use the minimal volume of a suitable solvent to disrupt specific interactions established between the sorbent and the target analyte. Although its optimisation is not as important as for the previously described steps, the lesser the volume used in the elution process, the higher the preconcentration of the sample. The most widely used solvents to perform this step are polar organic solvents such as methanol or acetonitrile because, in most cases, the interaction between the target analyte and the polymer is based on hydrogen bonding and these solvents can effectively disrupt this kind of interaction. However, to decrease even further the volume used in the eluting solvent, a common practice is the introduction of a low percentage (usually less than 5% v/v) of an organic modifier into the eluting solvent. The reason for that is to favour disruption of the hydrogen bond established between the target analyte and the sorbent since these organic modifiers can establish a very effective hydrogen bond with the functional monomers present in the MIP. In addition, this organic modifier is present in the eluting solvent in excess and, apparently, this solvent weakens the interaction of the affinity sites in the polymer with the target molecule, thus displacing the analyte retained on the MIP to the eluting solvent [92]. The most widely used organic modifiers so far have been acetic acid [93-95] or its analogue, trifluoroacetic acid [96-98].

Once all the MISPE steps have been optimised, a problem can sometimes occur, especially when using highly sensitive detection systems such as MS. This is known as bleeding of the cartridge. This problem, as mentioned previously, is a consequence of an inefficient removal of the template molecule used during the synthesis of the polymer once the MIP is obtained. Therefore, when the sorbent is used in a MISPE protocol, remaining template molecules might still elute from the cartridge, thus masking the final result obtained.

In these cases, the mostly adopted solution is the use of an analogue of the target molecule in order to obtain high selectivity for the molecule intended. In this case, if bleeding problem occurs, since the template molecule used to synthesise the MIP is different to the target, the bleeding of the template molecule do not mask the quantification o the target analyte when they are determined by a chromatographic technique.

All the above-mentioned stages are essential steps in any MISPE protocol, although, as stated previously, when loading the MIP with an organic solvent, the clean-up step can sometimes be avoided.

There are basically two distinct protocols to perform all these stages: MISPE *off-line* and MISPE *on-line* generally coupled to liquid chromatography (LC).

MISPE *off-line* is the mostly used technique. In this case, the useful particles are generally suspended in a solvent and then poured into an empty polyethylene cartridge. As in the conventional SPE cartridge, the particles are held between two

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frits to avoid any losses. The cartridge is then connected to a SPE manifold and the sample is percolated through by negative pressure. The most widely used mass of sorbent for MISPE applications normally ranges from 40 to 200 mg of suitable particles [52,72] . Their particle size and shape depends on the polymerisation approach taken during the synthesis of the MIP. In the synthetic protocols aiming to deliver spherical particles, the particles obtained are ready to be used, with no need for any further processing [63]. In the case of Traditional Polymerisation, in which MIPs are obtained in a monolithic form, there needs to be further processing so that the particles for MISPE applications are in the range of between 20 and 60 µm [39]. This range is the right balance because the particle size is low enough to enable both a proper flow and mass-transfer of the analytes present in the mobile phase on to the sorbent.

For *on-line* MISPE coupled to LC, the sorbent is placed in a precolumn, which is coupled to the chromatographic system. In this case, and because of the set-up used, the most difficult stage to carry out is the elution step because the eluting solvent is the mobile phase used in the chromatographic system. Because of this, the mobile phase must efficiently elute all of the analytes retained on the sorbent. If the analytes are retained on the MIP and are not eluted within the front volume of the mobile phase, not all of the compound will reach the column at the same time, thus broadening and overlapping the chromatographic peaks obtained.

Once loaded the cartridge, the clean-up step is also performed by pumping a suitable cleaning solvent through the column. Again, as for *off-line* MISPE, depending on the solvent used in the cleaning step, a previous drying of the cartridge is required before the eluting step. The most common practice of drying the cartridge is by flushing air through the cartridge. However, due to the higher complexity of *on-line* MISPE set-up, this step is more difficult to perform than for *off-line* MISPE.

There are several studies reporting on the use of on-line MISPE applications [66,67]. Moreover, in 2000 our group was the first one to report on the use of an *on-line* MISPE-LC to selectively extract 4-nitrophenol from river water [99]. For this set-up, a schematic representation is depicted in Fig. 11.

As depicted in Fig. 11, at least a 6-port valve is required in order to allow two pumping systems to run in parallel. Both the loading and the cleaning of the MISPE cartridge are carried out (continuous line) when the valve is set in the loading position. In this position, the column and precolumn used in this set-up are not yet connected and the sample and cleaning solvent do not pass through the chromatographic system. When switching the valve to its injection position, the mobile phase of the chromatographic system passes first through the MISPE precolum in order to elute the analyte retained and then through the chromatographic column for their separation (dashed line).

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In *on-line* MISPE applications, the amount of sorbent used is smaller than in *off-line* applications and, therefore, the volumes of sample generally analysed in *on-line* applications are lower. However, when comparing the two protocols using the same mass of sorbent, the limits of detection for *on-line* applications are lower than those obtained for *off-line*. This is because, for *on-line* MISPE protocols, all of the analyte present in the sample is preconcentrated in the cartridge and further quantified, whereas for off-line applications only a part of the total amount of analyte present in the sample is actually quantified.

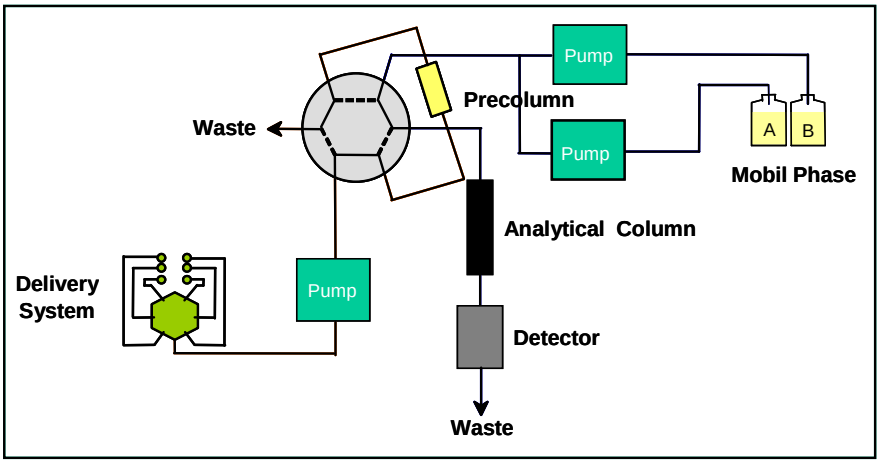

Fig. 11. Set-up of a MISPE *on-line* protocol coupled to LC.

1.4 MIPs for other sorptive extraction techniques

In order to further exploit the use of MIPs in selective extractions, several studies applying imprinting technology to different extraction techniques have appeared over the last few years. These studies looked at combining both the advantages offered by MIPs and those offered by these different techniques.

These new applications are focused on sorptive extraction techniques, such as solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE) and matrix-solid phase dispersion (MSPD) techniques.

Solid-Phase Microextraction (SPME) appeared in 1990 [100] and, since then, many different studies have looked at using this simple method to extract different analytes from many different matrices. When comparing this technique to other commonly used extraction techniques, such as SPE or liquid-liquid extractions, SPME can extract the analyte of interest in a very time-efficient way and it is almost free of solvent. As with any sorptive extraction technique, SPME is based on a partition of the analytes between the media they are in and a solid support. Therefore, the

coating of the fiber is very important for the efficiency of the SPME protocol developed. Another advantage of this technique is that the fiber can be thermally desorbed on the GC system. However, thermally unstable compounds can be desorbed using an organic solvent and be further quantified by LC.

Despite the fact there are several commercially available fibers to choose from, there is still a lack of fibers for extracting different compounds with different functionalities.

Under these circumstances, different studies have been done in which the authors synthesised their own fibers and made use of MIPs as sorbents on those fibers to take advantage of the selectivity offered by MIPs.

When synthesising SPME fibers and due to the final format required, the best-suited polymerisation technique to obtain the desired fiber is Traditional Polymerisation. Therefore, all of the fibers obtained so far have been synthesised using this approach. In this case, the monolith obtained from Traditional Polymerisation is attached to a solid support that, in turn, is responsible for delivering the shape of the final product required. To do this, it is crucial to do a proper sililation of the support because the success of obtaining the desired fiber will depend on this step.

Since the synthesis of home-made SPME fibers is a rather new technique, there is no uniformity on how to obtain the fibers and there are several different supports described in literature to which fibers have been attached. The most widely-used support has been the use of a commercial fiber in which the coating surface has been etched away, as Hu *et al*. [101] or Prasdad *et al*. [102] reported. Another way to obtain SPME fibers is, as Turiel *et al*. [103] or Djozan *et al.* [104] reported, by using a glass capillary, filling it with the polymeristion mixture and inducing the polymerisation process inside. Another support that can be used, as Koster *et al*. described [105], is a hollow tube, such as an optic fiber, to which the MIP is attached.

A common drawback for most of the homemade SPME fibers described above is the fragility of the final material produced. Since these fibers are highly cross-linked monolithic polymers, extra care is required when handling such materials to ensure that material produced is not broken.

All of the above-mentioned SPME fibers have been successfully applied to many different matrices and very good results have been obtained for each of them. For instance, Hu *et al*. [101] applied a fiber obtained to selectively extract five triazines from corn, lettuce, soya-bean and soil samples. Recoveries of over 75% were achieved in all the cases, with limits of detection ranging from 0.012 to 0.090 μg L^{-1} when LC-UV was used as the separation and detection system. Prasdad *et al*. [102] also extracted ascorbic acid at concentration levels ranging from 0.11 to 65.3 μg L^{-1} from human serum with recoveries of 100% without any sample pretreatment and quantified it using differential-pulse, cathodic stripping voltammetry and a hanging

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mercury drop electrode as the separation and detection technique. Djozan *et al.* [104] also extracted seven triazinic compounds with recoveries of over 90% in most cases from tap water, onion and rice samples. These compounds were detected with limits of detection ranging from 20 to 90 µg L^{-1} when using GC-MS as the separation and detection system.

Turiel *et al*. [103] also successfully applied their homemade SPME fiber to extract triazinic compounds from food samples at a concentration level of 17 µg Kg^{-1}, using LC-UV as the separation and detection system. In this case and contrary to all of the fibers previously mentioned, the fiber obtained was very flexible. This demonstated that fragility of the fibers can be overcome by carefully selecting the appropriate amount of compounds involved in the polymerisation process.

Fig. 12 schematically shows the protocol developed by Turiel *et al*. in the synthesis of the SPME fiber. It also shows a picture of the degree of flexibility achieved for this fiber.

Firstly, capillaries were cut at 30 cm long and then, every 5 cm, windows of 1 cm long were produced in the capillary by pulling out the coating surface from the capillary. Afterwards, the capillary was filled with the polymerisation mixture, the ends were sealed and polymerisation was induced. Once polymerisation was completed, the capillary was cut at 5 cm long and, in order to reveal the polymer produced, the capillary was removed by immersing it in NH_4HF_2. The fibers were then ready to be used. The picture also shows the high degree of flexibility for the fiber synthesised since the polymer obtained can easily be bent without breaking.

Stir Bar Sorptive Extraction (SBSE) is another technique that is attracting interest in using MIPs as coating agents. SBSE is an extraction technique that was first introduced by Baltussen *et al.* [106] in 1999 and was mainly aimed to increase the mass of sorbent used in SPME fibers. This technique is based on the use of a magnet covered by a jacket of polydimethylsiloxane. This magnet is left in a solution under vigorous stirring and the compounds present in solution are retained on the covering of this magnet. Desorption of the compounds extracted is performed using very low volumes of organic solvent or thermally in GC equipment.

As with SPME protocols, the ease of the protocol and the high recoveries obtained for different compounds make this extraction technique very attractive for routine use. Due to the fact that only apolar coatings based on polydimethylsiloxane are commercially-available for this technique, to broaden the use of SBSE to more polar compounds some authors have developed their own sorbents [107,108]. Moreover, due to the high selectivity displayed by MIPs and the ease of obtaining these polymers, several studies exploiting the use of MIPs as coating sorbents is SBSE protocols have been developed [109-111].

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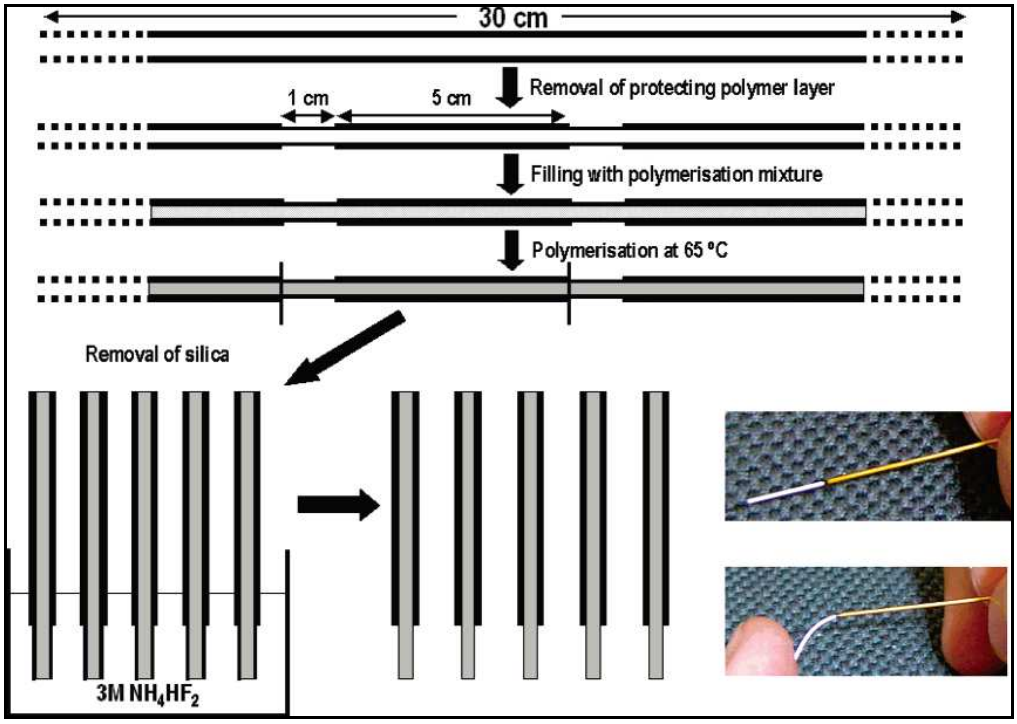

Fig. 12. Synthetic process for obtaining the imprinted fiber and a picture showing its flexibility [103].

For example, Zhu *et al.* [109] used a method for synthesising a stir-bar coated with a MIP from a conventional stir-bar. In this case, the authors prepared a mixture of Nylon-6 (30%), L-glutamine as the template molecule (3%) and formic acid (67%) and a stir-bar was dropped inside this colloid solution. Once the stir-bar had been coated with the colloid solution, it was transferred to a water solution in order to induce gelation of the MIP. After gelation of the polymerisation mixture on the stir-bar, it was ready to be used as an extracting agent. The stir-bar obtained was able to selectively rebind the target analyte from a mixture of five different closely-related aminoacids. The authors also described the synthesis (using the same protocol) of a stir-bar coated with a MIP using monocrotophos as the template molecule [110]. In this case, the authors were able to extract four different pesticides from soil spiked at 100 µg Kg^{-1} with recoveries ranging from 71% to 92%.

Another example of a SBSE covered with a MIP was reported by Hu *et al.* [111]. In this case, the authors synthesised a polydimethylsiloxane stir-bar in the presence of the template molecule. To achieve this, the authors used an iron bar held inside a glass tube and sililated the outer surface of the glass. Once the glass had been sililated, it was inserted in a previously prepared mixture of polydimethylsiloxane, β-cyclodextrin as the template molecule and all the typical compounds involved in the synthesis of a normal stir-bar and template molecule. This step was repeated several

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times until the desired thickness of the stir-bar was achieved. With this modified polydimethylsiloxane, the authors could selectively extract four different estrogen compounds from 5 mL of water samples, reaching limits of detection for all of them ranging from 0,04 to 0,11 μg L^{-1} using LC and UV as separation and detection system, respectively.

Matrix Solid-Phase Dispersion (MSPD) is another extraction technique that was introduced by Barker *et al*. [112] and it is based on the same basic principle as SPE. The main difference between this technique and other SPE related techniques is that matrix solid-phase dispersion (MSPD) can perform the extraction directly on solid, semisolid and viscous samples. It is also possible for this technique to be performed on liquid samples [113].

MSPD involves very simple and easy steps that make it possible to obtain an efficient extraction from a sample while considerably diminishing the use of organic solvents.

The first step involved is to blend together the sample of interest and the sorbent used in the extraction process. Since MSPD is mainly used for extracting solid samples, the sample of interest is grounded and blended with a suitable sorbent in a mortar.

The next step is to pack this mixture into an empty reservoir, such as an empty column, a SPE cartridge or a syringe.

Once the mixture is packed, there are two different ways to extract the analytes of interest, depending on their affinity towards the sorbent used. On the one hand, if there is a high retention of the analyte of interest on the sorbent, a solvent can be percolated through the cartridge to remove the compounds not as strongly retained on the sorbent as the target analyte. On the other hand, if the analyte of interest is not so strongly retained on the sorbent, a solvent able to disrupt only the weaker interactions can be used and, in this way, the compounds retained on the sorbent are the interfering compounds. In all of these cases, the extract finally obtained contains the analyte of interest, free from most of the interfering compounds in the sample.

Due to the simplicity of the MSPD extraction method, any commercially available sorbent can be used. The sorbents most widely used have been those based on C_8 and C_{18} bonded silica supports but there is a trend nowadays to use different sorbents with different functionalities to detect polar compounds. For example, Morzycka *et al*. [114] used Florisil, a magnesium-silica sorbent for preparative chromatography, to determine 12 insecticides by GC-NPD in 0.5 g of honeybees. The method developed enabled detection and quantification at concentration levels ranging from 0.03 to 1 mg Kg^{-1}. Another example was reported by Garcia *et al*. [115] in which the authors also used Florisil to determine organophosphate compounds in indoor dust samples by using MSPD and GC-NPD. In this way, the authors detected levels of 1 mg Kg^{-1} of the analytes of interest.

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As with other extraction techniques, some authors have also tried to combine the advantages that MIPs offer for selective extraction with the simplicity of the present technique. To this end, Guo *et al*. [116] reported on the determination of chloramphenicol in 100 mg of fish tissue samples and, when using LC-UV as the separation and detection technique, the limit of quantification was 3.4 µg Kg^{-1}. In this case, the recoveries of the target analyte in a MSPD when using the MIP as sorbent were higher than when using any other commercially available sorbent (C_{18} and Attapulgite, respectively). The repeatability was also high when using the MIP as a sorbent.

Another interesting example was reported by Sun *et al*. [117] on the determination of six fluoroquinolones from serum samples. This study proves the feasibility of performing a MSPD extraction directly on a liquid sample. In this case, the authors blended the serum sample and the MIP synthesised for the target analytes directly and did a clean-up step of the sample with water. In this way, the authors could detect the compounds of interest from 100 µL of serum sample at concentration levels as low as 0.05 mg L^{-1} with recoveries ranging from 74% to 114% for all of the compounds of interest when using LC-UV as the separation and detection system. Again, as in the case of Guo *et al*., the authors compared the performance of a MIP in a MSPD extraction to the same extraction using either C_{18} or Fluoisil as a sorbent. Recoveries were shown to be far higher when using the MIP than with any of the commercially available sorbents.

1.5 MISPE applications

The main advantage of a MISPE protocol is the high selectivity that this protocol brings to the isolation of the target analyte, thus improving its further quantification. The high selectivity displayed by MIPs arises, as explained previously, because the synthesis of the sorbent is carried out in the presence of the template molecule. In this way, cavities complimentary in size, shape and functionalities are formed within the polymer that perfectly accommodate the target molecule. Due to the high complimentarity of the active sites on the target molecule, this molecule is strongly retained on the sorbent rather than any other molecule present in the sample. As a consequence, a strong clean up of the sample can be performed with minor losses of the target molecule. In this way, molecules retained on the MIP by non-selective interactions can easily be removed while keeping the target analyte and, therefore, cleaner extracts of the sample can be obtained.

However, there are many cases where, apart from the target molecule, some other structures closely related to this target molecule can also be retained. This effect is known as *cross-selectivity*. In some cases, the cross-selectivity displayed by the MIPs is highly desirable since it broadens their applicability because molecules other than the target molecule can also be retained on the MIP and further quantified. This can be explained by considering that similar compounds within the same family

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share common structural features that, when the target molecule is retained through these features, causes them to be retained also. This is the case, as Carabías-Martínez *et al.* reported [118], with the detection of triazines and their dealkylated and hydroxylated metabolites from river water or, as Caro *et al*. also reported, on the extraction of 4-chloro-substituted compounds and 4-nitrophenol [46] or fluoroquinolonic compounds from urine and tissue samples [119], amongst others. Another clear example of induced cross-selectivity in the MIP is when using analogues to the target molecule for which the MIP is intended to be selective for. Although the reasons for using such analogues are very different than just inducing cross-selectivity to the MIP, this is an obvious case in which a different molecule than the one used in the synthesis of the MIP is retained on the polymer. In this case, the analogue used is very similar in size, shape and functionalities to the molecule intended to be retained on the MIP. This approach was adopted by Theodoridis *et al.* [120] when synthesising a MIP for the selective extraction of scopolamine from urine and serum samples. The authors used hyoscyamine instead as the template molecule during the synthesis of the MIP.

An elegant way to take advantage of the cross-selectivity that MIPs exhibit is by using this capability to overcome the previously mentioned bleeding problem. To this end, the MIP is synthesised by using, as a template molecule, a very closely related structure (also known as dummy template) to that molecule the MIP is intended to be selective for. Therefore, very similar cavities with the active sites properly distributed within this cavity are also generated. Once the MIP is synthesised, even if the bleeding problem arises, and since the template and the target molecule are different, the target molecule can easily be quantified because it will have a different retention time in the chromatogram. For example, several MIPs obtained using template analogues have demonstrated good recoveries of the target analyte [83]. However, the use of a different molecule during the synthesis of the MIP can sometimes offer lower recoveries for the target molecule [121].

A good example of the improvements achieved after performing the MISPE technique is shown in Fig. 13 for the extraction of several triazines from potato samples. Additionally, the effect of the cross-selectivity of the polymer can also be observed [63]. Chromatogram (a) shows the complexity of the sample and all of the different compounds present in it, whereas chromatogram (b) shows the effect of the efficient removal of all of the non-selective analytes retained on the MIP after cleaning the MIP with 1 mL of toluene. By comparing these two chromatograms, it can be seen that the clean-up step highly facilitates the isolation and quantification of the analytes of interest. Another interesting feature observed in this case is the cross-selectivity displayed by the MIP. In this case, the authors used procaine as a template molecule. Since triazine compounds share the same core structure, not only was the target analyte retained on the MIP but so were many other structurally related compounds, enabling proper quantification of all of them.

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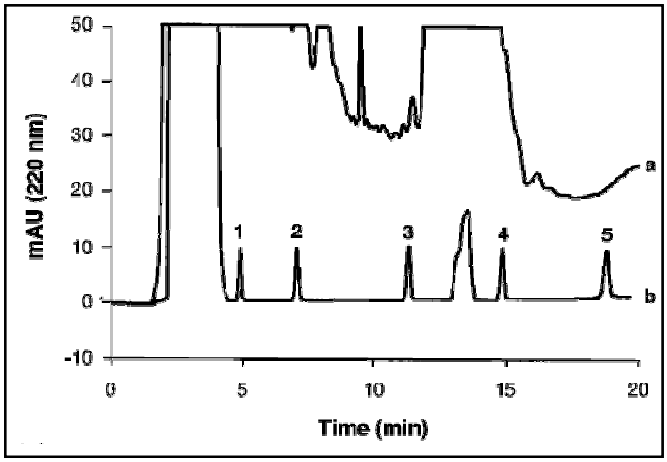

Fig. 13. Chromatograms obtained before (a) and after (b) the MISPE protocol of potato extracts spiked at 20 µg Kg^{-1} of different triazines. (1) desisopropylatrazine, (2) desethylatrazine; (3) simazine; (4) atrazine; (5) propazine [63].

The previous example is evidence of the many different matrices in which the selectivity of MIPs has been exploited and, as expected, a pre-treatment of the sample must be performed in some cases. This is the case with highly complex matrices, such as blood, plasma or serum, in which deproteinisation of the sample is required to avoid blocking the cartridge. For example, this was the sample pre-treatment required for extracting lamotrigine from serum samples [122] or afluzosin from plasma [95]. There are also some other liquid extractions for different purposes reported in literature, such as the extraction of Sudan I from red chilli powder [123], the extraction of domoic acid from blue mussels [124], or the extraction of phenylurea herbicides from plant samples [125].

Another common practice before MISPE is to extract the target analyte from solid samples using different extraction techniques as, for instance, pressurised liquid extraction (PLE). For PLE extractions, the sample is extracted using a suitable solvent under both high pressure and temperature. With this technique, a more effective extraction is performed and the volume of solvent needed is reduced. This was the extraction technique used in the extraction of nerve agents from soil samples [126] or zearalenone from cereals [127] and the extracts obtained were further percolated through a MIP to obtain the desired selectivity. Another commonly-used extraction technique is ultrasonic assisted extraction. This was the case of the extraction of paraben compounds from soil samples [128].

When MIPs first became used, it was believed that the best solvent to load the cartridge with was the same one used as a porogen during its synthesis. Therefore, since MIPs are generally obtained using organic solvents, polar and protic solvents

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were avoided. There are two main reasons why most of the selective extractions have been performed using organic solvents. On the one hand, there are some MISPE applications that, when the MIP is loaded using aqueous matrices, the analyte is not retained at all [129]. In these cases, release of the analyte might be due to the fact that the high polarity of the target analyte makes its extraction from water difficult. It could also be for the reason that, since water is a protic solvent, the selective interaction responsible for the retention of the target analyte on to the MIP is disrupted. On the other hand, since organic solvents are normally used as solvents for performing the extractions of either tissues [119,130], vegetables [40,131] or biological samples [122,132], the extract obtained can be directly applied to the MISPE protocol. Moreover, and as stated previously, when the MIP is loaded using organic solvents, in most of the cases the clean-up of the sample is not required since the organic solvent prevents hydrophobic interactions on the MIP to be established.

However, over the last few years, an increasing number of applications using aqueous matrices such as urine, sewage water [133] and water from either lakes [134], rivers [46,67,135] or taps [38,136] have appeared. In all of these applications, the analytes of interest were successfully extracted regardless of whether they were in a polar or protic solvent. This can be explained by considering, as Claude *et al.* [137] or Fan *et al.* [138] reported, that retention of the target analytes on to the MIP are not through selective interactions when the loading solvent used is water. In these cases, the main interactions between the target analyte and the MIP are non-specific interactions such as hydrophobic or Л-Л interactions and the selectivity of the method is achieved during the clean-up step. In the clean-up step, all the molecules that are not able to establish a selective interaction with the polymer are removed by the organic solvent. The only molecules that remain retained on the sorbent are those that can establish selective interactions.

Studies that have reported using MIP in the selective extraction of compounds of concern from water-based matrices clearly point out that the limitation of the application of MIPs using water is being overcome.

Although the main goal for MISPE techniques is the selectivity achieved, since MISPE is a special case of SPE, preconcentration of the sample is also highly desirable. To this end, there are some papers described in literature in which an important preconcentration factor of the sample has been obtained. This is the case with Le Noir *et al.* [139], who described the extraction of 17-β-estradiol at 2 μg L^{-1} from 2 L of effluent water from a waste water treatment plant; or Zhu *et al.* [140], who reported on the extraction of four different pesticides from 1 L of river and tap water; or our group [46], who reported on the extraction of naphthalene sulfonates from 1 L of river water.

However, the generally low breakthrough volume exhibited for the MIP makes these sorbents more suitable for selective extraction rather than as a

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preconcentration technique. However, when aiming to extract the analyte of interest from a large volume of sample and the MIP does not allow the direct extraction of this analyte, a common practice is to use a commercially-available sorbent in order to preconcentrate the sample and, once this sorbent is eluted, to further percolate this fraction through the MIP to achieve the desired selectivity [118,137].

Over the last few years, MIPs have become widely accepted as selective sorbents in MISPE applications and interest in them goes beyond strict research purposes. Nowadays there are commercially available MIPs for some interesting applications. Some examples of the use of commercially-available MIPs are those reported by Widstrand *et al.* in which the authors reported on the use of a commercial MIP for the detection of β-agonist in calves urine [141]. Lara *et al.* also reported on the use of a commercially-available MIP for the selective detection of triazines from urine [142], or as Mohamed *et al.* did on the detection of four 5-nitroimidazole compounds from egg samples [84]. In all of these cases, the MIPs were obtained from MIP Technologies [143]. However, there is still a lack of commercial availability for these kinds of sorbents and the only feasible way to obtain them is by in-house production.

Due to the nature of the compounds studied, most of the MISPE applications have been performed using LC and, because of the high selectivity that MIPs offer over other SPE sorbents, the most widely-used detection system so far has been UV. The improvement, achieved when using a MIP as a SPE sorbent compared to a commercially-available sorbent, enables the detection of the analyte of interest using this most commonly-used detection system [90]. However, there are some cases in which, due to the high complexity of the sample and the low concentration level at which the analyte of interest in present, not even the big improvement in selectivity achieved by MIPs is enough to quantify the analyte using LC and UV detection systems. Therefore, the use of a more sensitive detector, such as a mass-spectrometry detector, is required [144,145]. So, when performing targeted analysis, the best option in order to obtain the highest selectivity is to probably use MISPE and LC-MS or even LC-MS-MS.

Since MISPE extracts are usually analysed using LC, a common drawback when using LC-MS with electrospray ionisation (ESI) is the typical ion-suppression arising from the huge number of compounds present in the samples. In these cases, the use of MIPs offer, once more, an added value compared to the conventional sorbents because - due to the fact that extracts obtained by using MIPs are cleaner - the ionic suppression is highly decreased, leading to lower limits of detection. To this end, Demeestere *et al.* [145] compared the ionic suppressions obtained using a non-selective sorbent and a MIP for the selective extraction of antidepressants in environmental water samples. As expected, the use of the MIP decreased the ionic suppression of the sample significantly and allowed lower detection limits to be achieved.

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1.5.1 Targeted sorbents: useful counterparts for selective extractions

After describing the mostly used synthetic protocols and the main advantages and drawbacks for each of them as well as putting in context the main advantages that MIPs offer when performing selective extractions, we considered that summarising all the information used over the last years could provide a useful overview on the latest trends and applications of MIPs to SPE.

In the following paper there is a discussion on the main synthetic aspects involved in the production of MIPs as well as the main MISPE applications considering the most relevant papers recently published.

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Targeted sorbents: useful counterparts for selective extractions

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In this paper we review and discuss the mostly commonly used synthetic protocols for obtaining molecularly imprinted polymers (MIPs) and highlight the main advantages and drawbacks of each protocol. We then evaluate the most recent applications of MIPs and also focus on the mostly adopted strategies for overcoming the common inconvenient encountered when applying MIPs in different matrices.

We also highlight the different advantages that MIPs offer when they are used as sorbents in SPE protocols for performing highly selective extractions and the advantages these sorbents bring over the most commonly-used SPE sorbents by reviewing some of the latest studies reported in literature.

Keywords: Molecularly imprinted polymer; Solid phase extraction; Polymerisation techniques; Environmental analysis; Selective extraction

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1. Introduction

Scientists all-over the world are developing new technologies and re-inventing existing ones in the never-ending quest to obtain lower concentration levels of different analytes in many different and highly complex matrices.

When trying to detect low concentrations of the analytes of interest in real samples, which in some cases are extremely complex, preparing the sample prior to instrumental analysis is almost unavoidable.

In the case of liquid samples, the most commonly used sample pretreatment is solid-phase extraction (SPE). From the beginning of SPE sorbents, most of research has focused on developing new sorbents which enabled large samples volumes to be extracted in order to reach low limits of detection.

However, due to the high complexity of the sample, quantification of the analytes in some cases was very difficult. Therefore, the research shifted its focus to more selective extractions with the result that nowadays a lot of effort is being expended on developing new sorbents [1]. The shift to more selective extractions has been forced because for any given SPE protocol many other compounds in addition to the analytes of interest are preconcentrated on the cartridge. The best way to overcome this drawback is by using sorbents which enable selective extractions. There are several different ways of performing such selective extractions. One option is to use immunosorbents (IS). Because of their high selectivity, IS can perform very selective extractions but, due to both difficulties during their synthesis and their conditions of use [2], their applications are very limited. Another option is to use mixed-mode sorbents; this is a new trend in the SPE sorbents and has led to sorbents that can trap analytes both by reversed phase and ionic interactions, therefore enabling the quite selective extraction of compounds with similar ionic character [3]. Another possibility is to use molecularly imprinted polymers (MIPs). MIPs overcome the limitations inherent in the synthesis and use of IS whilst maintaining a high degree of selectivity and the simplicity of the synthetic process of polymeric sorbents.

The first use of MIPs was reported by Ali Sarhan *et al*. in 1972 [4]; however, it was not until 1983 that Vlatakis *et al*. [5] published a paper which led to the widespread application of MIPs in many different fields.

MIPs are specially designed sorbents which display enhanced selectivity towards a certain structure (or to a very closely related structure). Due to their inherent properties, MIPs have been widely used in many different applications such as sensors [6] enantiomeric separations [7] and catalysts [8].

Furthermore, ever since Sellergren [9] first reported the use of MIPs as sorbents specifically in SPE, their application in this field has become widely accepted because of their highly selective extraction of the analyte of interest. The widespread acceptance of MIPs as SPE sorbents has led this practice to become known as molecularly imprinted solid-phase extraction (MISPE).

In MISPE, the high selectivity of the sorbent towards a given structure enables this structure to be selectively retained on the sorbent while the other compounds are not retined, resulting in the desired compound being extracted from most of other compounds present in the sample. Because MIP sorbents are so highly selective, they have also been applied as home-made sorbents in other extraction techniques such as solid-phase microextration fibres (SPME) [10] and stir-bar sorptive extraction (SBSE) [11]. More recently, Chimuka *et al.* [12, 13]

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reported a new liquid-liquid extraction technique in which they used a membrane in the interface of the two solvents and incorporated some MIP particles into the extracting solvent.

Since the appearance of MIPs, various papers have reviewed different aspects of imprinting technology. Probably the most representative of these papers is a review by Alexander *et al*. [14] in which the authors describe nearly 1500 references covering all the different areas as well as the fundamental aspects of imprinting technology from its beginnings up to 2003. In addition to this review, other reviews have appeared describing certain aspects related to the use of MIPs in MISPE applications. Examples of the most recent are Pichon *et al*. [15], who reviewed sample treatment using MIPs, and Tamayo *et al*. [16], who reviewed attempts to improve the performance of MIPs. Apart from these, Haginaka [17] recently reviewed particulate and monolithic imprinted materials and some examples of how these could be applied, and Caro *et al*. [18] reviewed the extraction of the analytes of interest from environmental and biological samples.

In the present paper we explain the main approaches in the synthesis of MIPs and the latest trends in MISPE protocols that have emerged over the last few years.

2. Synthesis of MIPs

MIPs are polymeric sorbents obtained by polymerising suitable monomers in the presence of a template molecule. One way to classify them is in terms of the interaction between the functional monomer and the template molecule in the synthesis and in the rebinding steps. Depending on the nature of this interaction, MIPs can be classified according to whether they are obtained using the covalent, semi-covalent or non-covalent approach. The covalent approach is not used for synthesising MIPs to be applied in MISPE due to the disadvantages in both the synthesis of the MIP and rebinding of the target molecule. The most widely used approach to synthesising MIPs for use in MISPE protocols has traditionally been the non-covalent approach, although in recent years several papers have emerged reporting the use of the semi-covalent approach [19, 20]. Reporting on both approaches, Cacho *et al*. [21] compared a MIP obtained using the semi-covalent approach with one that they had previously obtained using the non-covalent approach [22] to determine their respective effectiveness in the selective extraction of triazines from real samples. From this comparison the authors concluded that the MIP obtained using the semi-covalent approach yielded a better clean-up of the sample than the MIP obtained using the non-covalent approach.

The difference between the semi-covalent and non-covalent approaches is the interaction between the template and the functional monomer before the polymerisation process which is covalent for the former and non-covalent for the latter. Because each approach causes a different interaction during the synthesis of the polymer, a different template molecule:functional monomer ratio is needed to ensure the best imprint on the final polymer. The template molecule:functional monomer ratio normally used in the semi-covalent approach is rather low and is generally 1:1 or 1:2 [19, 21], whereas for the non-covalent approach, ratios typically range from 1:4 to 1:8 depending upon the complexity of the template and the affinity of the functional monomer to the template. Since the choice of ratio is not a trivial decision, some authors have made exhaustive studies of the best monomer composition for the intended MIP [23].

Choosing the right functional monomer is very important because this will determine, on one hand, the stability of the complex formed before and during the polymerisation process and, on the other hand, the subsequent ability of the MIP to selectively interact with the target molecule. For most functional monomers, the selective retention of the analytes onto the polymer can be established either by H bond or ionic interaction, depending upon the solvent and the pH of the sample being percolated.

Although there are many different commercially available functional monomers to choose from, the most widely used to date have been methacrylic acid (MAA) and 4-vinylpyridine (4-VP), as can be seen in Tables 1, 2 and 3, which summarize some of their most recent applications. Due to its nature, MAA is the preferred monomer for interacting with basic compounds, whereas 4-VP is preferred for acidic compounds. However, since both compounds can establish a strong hydrogen bond, both have been used for extracting either acid or basic compounds. For example, MAA has been used to extract diverse substances such as antibiotics [24], antiepileptic drugs [25] or doping agents [26], whereas the use of 4-VP has also been recently reported for extracting barbiturates from river water [27], 2,4-nitrophenol [28] from river water and enrofloxacine from milk samples [29].

To further increase the selectivity of a MIP, it is the common to synthesize the functional monomer according to the functionalities of the target molecule. In such cases, the functional monomer synthesised generally has several interaction points with the template molecule. Sellergren *et al.* have extensively reported on this practice. For example, they synthesised specially designed functional monomers for the selective extraction of 6 penicillines [30] or riboflavine [31] from aqueous matrices amongst others compounds. For example, Fig. 1 depicts the most likely interaction between the functional molecule 2,6-*bis*(acrylamido)pyridine and the template molecule. This

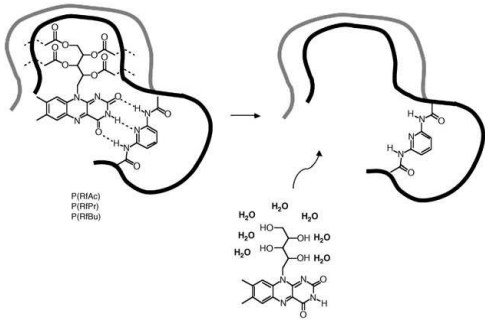

Figure 1. Cavities generated in a MIP using riboflavine tetraester as the template molecule and the home-synthesised 2,6-*bis*(acrylamido)pyridine as the functional monomer [31] (reprinted with editor's permission).

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Table 1. Applications of MISPE to foodstuff samples.

Template	Funct. Mon.	X-Linker	Analyte	Pol. Type	Matrix	Anal. Method	On/Off-line	Ref.
Cyromazine	MAA, HEMA	EGDMA	Melamine	Traditional	Milk	LC-UV, GC/MS	Off-line	[79]
17-β-estradiol	TFMAA	TRIM	17-β-estradiol	Precipitation	Milk	LC-UV	Off-line	[80]
Enrofloxacine	MAA, 4-VPy, DEAEM	EGDMA	Norfloxacin, Enrofloxacin, Ciprofloxacin	Suspension	Milk	LC-UV	Off-line	[29]
Ofloxacin	2-hydroxyehtyl methacrylate	EGDMA	Enrofloxacine, Ciprofloxacine	Traditional	Milk	LC-UV	Off-line	[87]
Bisphenol A	MAA, 4-VPy	EGDMA, TRIM	Bisphenol A	Traditional	Water, Milk	LC-MS	Off-line	[19]
Chloramphenicol	2-(diethylamino)ethyl methacrylate	EGDMA	Chloramphenicol	Suspension	Milk, Shrimp	LC-UV	Off-line	[48]
Tetracycline	MAA	EGDMA	Tetracycline	Traditional	Fish	Fl-Chem	On-line	[59]
Fumonisin B_1	2-diethylamino-ethylmethacry-late	TRIM	Fumonisin B analogues	Traditional	Bell pepper, Rice, Corn	LC-MS-MS	Off-line	[78]
Zeralone mimic	1-allylpiperazine	TRIM	Zeralenone	Traditional	Wheat, Rice, Corn, Barley	LC-Fluor.	Off-line	[64]
Propazine methacrylate	Semi-covalent	EGDMA	Simazine, Atrazine, Propazine	Precipitation	Soil, Potato, Corn	LC-UV	Off-line	[21]
Nitroimidazol analogue	MAA	DVB	4 5-nitroimidazoles	--	Egg	LC-MS	Off-line	[55]
Isoproturon, Linuron	MAA, TFMAA	EGDMA	Fenuron, Linuron, Metoxuron, Clortoluron, Isoproturon, Metobromuron	Silica particles	Potao, Pea, Corn		On-line	[50]
Tebuconazole	MAA, 4-VPy	TRIM	Tebuconazole	Precipitation	Cabbage, Pannage, Shrimp, Orange juice, Water	LC-UV	Off-line	[23]
o-phtalic acid	4-VPy	EGDMA	Domoic acid	Multi-step swelling	Blue mussels	LC-UV; LC-MS	On/Off-line	[47]
Linuron, Isporoturon	TFMAA	EGDMA	6 phenylureas	Precipitation	Potato, Corn, Pea, Carrot	LC-UV	Off-line	[88]
Chitosan	Allyl-chitosan	EGDMA	Chitosan, Quercitin, Morin	Traditional	Ginko biloba	LC-UV	Off-line	[20]
Pyrimethanil	MAA	EGDMA	Pyrimethanil	Silica particles	Wine	LC-UV	Off-line	[52]

functional monomer perfectly matches the imide functionality in the flavin ring systems, thus resulting in a very effective interaction between these two molecules.

The third party involved in the synthesis of a MIP is the cross-linking (X-link) agent, whose function is to deliver rigidity to the polymer. Although there are many different X-links to choose from, the most widely used is ethylene-glycol dimethacrylate (EGDMA), as can be seen in Tables 1, 2 and 3. An X-link is always used in larger quantity than either the template or the functional monomer and even though it is not as important as the functional monomer, choosing the right one is advisable because this can affect the hydrophobicity, especially when the samples of interest is a water-based matrice.

Several synthetic protocols have been developed to obtain these polymers, some of which will be discussed subsequently. However, since appearance of MIPs [4], the most widely used synthetic protocol has been *Traditional Polymerisation* (TP).

All the components involved in this polymerisation technique are dissolved in a low volume of a suitable solvent, also known as porogen, and left to polymerise, thus creating a monolith polymer. At this point it is also well worth noting that TP has been the technique of choice for synthesizing home-made fibres for solid-phase microextraction [10, 32] and stir-bar sorptive extraction [11, 33].

For MISPE applications, a polymer obtained by TP must be crushed, ground and sieved to obtain the useful particles. This process is laborious and time-consuming and produces low yields of the desired range of particles which are also irregular in size and shape (see Fig. 2A).

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Despite all these drawbacks, this is still the most widely used synthetic protocol because it is the most stream-lined and requires less specialized equipment and fewer organic skills. This polymerisation has been successfully applied to selectively extracting antibiotics from plasma [34], human urine samples [35, 36] and pesticides from water [37]. It has also been the polymerisation of choice for synthesising the above mentioned MIPs using a home-made functional monomer [30, 31].

Several polymerisation techniques have been developed for MISPE applications in order to overcome the irregularity in the size and shape of the useful range of imprinted particles and to reduce the time needed to obtain them. The aim of these techniques is to obtain spherical particles and, if possible, in a higher polymerisation yield than in TP. The spherical shape also improves both the packing of the cartridge and the mass-transfer of the analyte.

Tables 1, 2 and 3 show that *Precipitation Polymerisation* (PP) is the most widely used technique for obtaining spherical particles. This polymerisation technique delivers spherical particles of suitable size for MISPE applications, as can be seen in Fig. 2B. The basic principle of this approach is that when the polymeric chains growing in solution reach a certain critical mass, they precipitate from the solution. The particles thus obtained do not typically exceed 10 µm [38, 39] and are sometimes even in the sub-micrometer range which, for MISPE applications, is a limitation. A further drawback is that PP is not as robust as TP in terms of "imprintability", that is, the imprinting of any given template molecule. This is because the polymerisation conditions must be carefully chosen and because the template molecule has a great influence on the outcome of the polymeric particles. In this respect, several studies have shown that the presence of a template molecule during the polymerisation process has very different effects on the MIP and the non-imprinted polymer (NIP). For example, the presence of the template molecule can either decrease the polymerisation yield [40], increase the particle size [41] or have no effect at all [42].

Nevertheless, there are several studies where MIPs obtained using the PP protocol have been used as sorbents in MISPE applications. For example, Hu *et al*. have reported on the extraction of tebuconazole from food samples [23] and Martín-Esteban *et al*. have extensively reported on the extraction of several pesticides from vegetables [43, 44].

When we compared TP with PP we found that PP outperformed TP not only regarding synthetic issues such as polymerisation yield and particle uniformity but also in terms of sorbent capacities when sorbents were applied to MISPE [25, 41].

Another approach for obtaining spherical particles is by *Multi-step Swelling Polymerisation* (MSS). In this technique, pre-formed uniformly-sized seed particles are suspended in water and, after several additions of suitable organic solvents, the initial particles swell to a final size ranging from 5 to 10 µm (see Fig. 2C). Once the particles have swollen to the desired size, polymerisation is induced. Compared to PP, this approach is more robust when imprinting any given molecule because none of the compounds involved in the polymerisation process influences the process of obtaining spherical particles. However, as can be seen in Tables 1, 2 and 3, this kind of polymerisation has not been as widely used as either TP or PP due to its higher synthetic complexity. Nevertheless, Haginaka and co-workers have extensively reported on the use of this polymerisation technique to obtain imprinted materials and to extract barbiturates [45] and triazine herbicides [46] from river water samples. Kubo *et al*. [47] also used this technique to synthesise a MIP for extracting domoic acid from blue mussels.

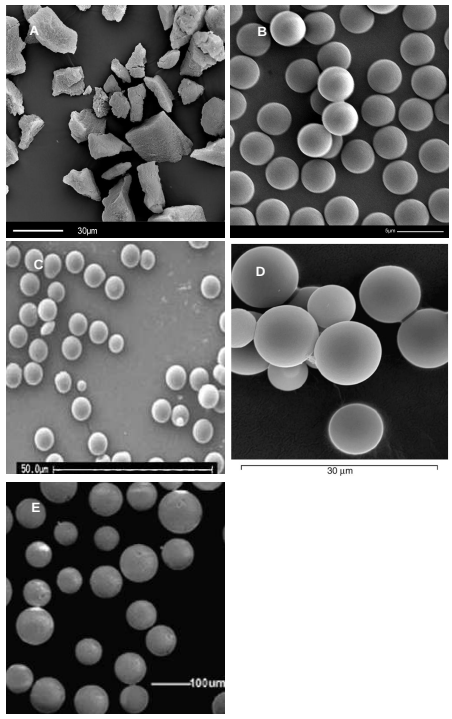

Figure 2. Particles obtained under: A) Traditional polymerisation, B) Precipitation polymerisation, C) Multi-step swelling polymerisation [91], D) Suspension polymerisation [48] and E) Grafting silica particles [50] (reprinted with editor's permission).

A different approach to obtaining spherical particles is to use *Suspension Polymerisation* (SP). In this case, all the components involved in the polymerisation process are dissolved together in an appropriate organic solvent. This solution is then added carefully to a larger volume of an immiscible solvent under vigorous stirring. The vigorous stirring causes the organic solvent containing the prepolymerisation mixture to form droplets (typically in the micron range) and then the polymerisation reaction is induced. The particles obtained under this protocol are, as Fig. 2D shows, also spherical and their size typically ranges between 10 and 100 µm. A feature common to both SP and MSS is that any given molecule can be used to deliver imprinted material and that the most commonly used dispersing agent in both cases is water. However, in both cases, the presence of water during the polymerisation process may jeopardize the

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Table 2. Applications of MISPE to biological samples.

Template	Funct. Mon.	X-Linker	Analyte	Pol. Type	Matrix	Anal. Method	On/Off-line	Ref.
Alfuzosin	MAA	EGDMA	Alfuzosin	Traditional	Plasma	LC-UV	On-line	[63]
Mycophenoli acid	4-VPy	EGDMA	Mycophenoli acid	Traditional	Plasma	LC-UV	Off-line	[65]
Alfuzosin	MAA	EGDMA	Alfuzosin	Traditional	Plasma, Soil	LC-UV	Off-line	[61]
Cefathiamidine	4-VPy	EGDMA	Cefathiamidine	Traditional	Plasma, Serum	LC-UV	Off-line	[34]
Diazepam	MAA	EGDMA	Benzodiazepines	Traditional	Hair	LC-MS-MS	Off-line	[89]
Lamotrigine	MAA	EGDMA	Lamotrigine	Traditional	Serum	LC-UV	Off-line	[62]
Ketamine	MAA	EGDMA	Ketamine, Norketamine	Traditional	Hair	LC-MS-MS	Off-line	[76]
Enrofloxacine	MAA, HEMA	EGDMA	4 quinolones	Traditional	Urine	LC-Fluor.	Off-line	[86]
Commercial	--	--	Atrazine + 3 metabolites	Traditional	Urine	UV	In-line	[54]
Oxfloxacine	MAA	TRIM	9 quinolones	Traditional	Urine	LC-UV	Off-line	[82]
Clomiphene (analogue)	MAA	EGDMA	Tamoxifen	Traditional	Urine	LC-UV	Off-line	[26]
Carbamazepine	MAA	EGDMA	Carbamazepine	Traditional	Urine	LC-UV	Off-line	[25,41]
		DVB	Oxcarbazepin	Precipitation				
Amoxicillin	MAA	EGDMA	Amoxicillin	Traditional	Urine	LC-UV	Off-line	[35,36]
Cephalexin			Cephalexin					
Dopamine	MAA, Acrylamide	EGDMA, MBAA	Dopamine	Traditional	Urine	LC-Fluor.	Off-line	[66]
Trimethoprim	MAA	EGDMA	Trimethoprim	Traditional, Suspension	Urine	LC-MS	Off-line	[49]

proper interaction between the template and the functional monomer. Although this kind of polymerisation technique has not been used as much others in recent years, some interesting papers have nevertheless appeared that describe the use of this technique to prepare a MIP for extracting enrofloxacine and other quinolone antibiotics from milk [29] and chloramphenicol from shrimp extracts [48].

Regarding the selectivity and capacity of the MIPs obtained by SP, Hu *et al*. [49] reported an interesting comparison between a MIP obtained by SP and a MIP obtained by TP. In this study, the MIP obtained by TP showed higher capacity and selectivity than the MIP obtained by SP and the authors accounted this fact to its higher pore size and the number of active sites formed within it.

Grafting is another polymerisation technique aimed at delivering spherical particles. In this case all the components involved in the polymerisation process are adsorbed within the silica particles before the polymerisation process starts. Once the polymer is formed, the silica is etched away to reveal a final product of spherical particles (as can be seen in Fig. 2E) which are in turn the specular image of the original silica particle.

In contrast to all the previously reported techniques, this one requires more synthetic skill and the use of corrosive solvents. Nevertheless, several reports have appeared over the last few years on the use of this technique for extracting herbicides from water and vegetables [50] 2,4-nitrophenol from water [51] and a fungicide from wine [52].

Together, the techniques mentioned above are the most widely used synthetic protocols that have been described in the literature for obtaining MIPs and for MISPE applications over the last few years. However, due to the wide acceptance that MIPs have had for performing selective extractions, these sorbents have also become commercially-available for performing routine selective extractions. Thus far, there are several to choose from and their application has also been described in literature. This is the case for the selective extraction of four non-steroidal anti-inflammatory drugs from waste water [53], atrazine and three of its metabolites from urine samples [54] and 4,5-nitroimidazol from egg [55], amongst others.

3. Applications of MIPs to solid-phase extraction

The main advantage that MIPs have to offer in SPE protocols is the dramatic improvement in the selectivity of the extraction. This high selectivity is because the

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sorbent provides stronger retention of the target analyte or closely related structures usually from the same family through the effect known as cross-selectivity. This enables cleaner extracts to be obtained thus facilitating the further quantification of the analytes of interest.

A good example of the improvement that MIPs offer compared to other sorbents was reported recently by González-Mariño *et al*. [56]. In this case the authors compared a commercial hydrophilic-lipophilic balanced SPE sorbent, a mixed-mode sorbent and a MIP. Their study showed that the hydrophilic-lipophilic balanced sorbent displayed the poorest results followed by the mixed-mode sorbent, whereas the MIP displayed the highest selectivity and accuracy, thus leading to lower limits of detection when extracting amphetamines from wastewater. Demeesteren *et al*. [57] also recently reported another interesting example of the advantages that MIPs offer over more widely used SPE sorbents. In this case, the authors compared the performance of a MIP with that of a hydrophilic-lipophilic balanced sorbent. As in the previous case, the MIP provided cleaner extracts from environmental waters which enabled better quantification of the antidepressants of interest. At this point, it is well worth noting that both of the MIPs used for performing the comparisons mentioned were commercially-available.

As in any SPE, the MISPE can either be performed *off-line* or *on-line* coupled to liquid chromatography (LC). The most widely used are the *off-line* mode because *on-line* applications are limited by the fact that the eluting solvent is the mobile phase of the chromatographic system. However, in the few years since our group described the first *on-line* MISPE-LC application [58], it has also been successfully used to extract barbiturates from river water samples [45], phenylurea herbicides from vegetable samples [50], tetracyclines from fish samples [59] and creatinine from blood [60].

To exploit the high selectivity of MIPs, MISPE is directly performed, when feasible, on the matrix of interest (mainly water-based samples). However, as in any SPE protocol, the direct application of the sample of interest in the MISPE protocol is not always feasible and a previous sample treatment is required. For example, Fig. 3 shows the improvement that can be achieved when using a MISPE in soil extracts. In this case, the authors extracted alfuzosin using a pressurized solvent extraction from soil samples and then percolated the extract through a MIP cartridge [61]. Prior treatment is also required for biological samples (see Table 2). In this case, the most commonly used protocol is to deproteinize the sample using acetonitrile to avoid blocking the cartridge before the MISPE. This procedure was followed by Sayed *et al*. [62] and Hugon-Chapious *et al*. [63] for the selective determination of lamotrigine and alfuzosin, respectively, in serum samples.

Other highly complex matrices where MIPs have also been widely exploited are food samples (see Table 1). Most of these cases require the analytes to be extracted with an organic solvent beforehand.

There are two main advantages of performing an extraction step on the sample. The first is that it provides the extracted fraction in the most suitable solvent for performing the MISPE. This can be achieved either by using the most suitable extraction solvent for the MISPE [59] or by evaporating this solvent to dryness and reconstituting the sample in a good solvent to perform the MISPE [64]. The second is that the final volume of the extracted fraction is generally low. This is an advantage for MISPE protocols because of the well-known generally low capacity of MIPs.

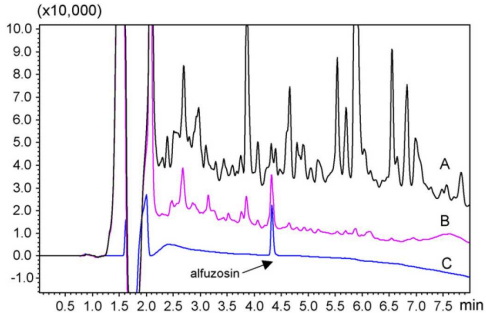

Figure 3. Comparison of chromatograms obtained (A) directly from soil extract spiked at 40 µg Kg^{-1} of afluzosin, (B) after the MISPE protocol and (C) direct injection of standard of afluzosin [61] (reprinted with editor's permission).

A common feature amongst both biological and food samples is the generally low sample volume required to perform the analysis. In the case of plasma samples, the volumes generally range between 0.5 – 2 mL [61, 65], whereas for some other complex matrices such as urine or milk, the volumes are normally in the low millilitre range [66, 67]. However, we have in-house experience of synthesizing a MIP that is selective for carbamazepine and that can extract the compound of interest and its main metabolite from 50 mL of human urine [41].

Even so, as Table 3 shows, the number of applications using large volumes of sample has increased over the last few years due to the number of extractions performed on environmental water samples [23, 68, 69].

Large volumes of these kinds of samples are required because the analyte concentration is generally low. For this reason, there are several studies in the bibliography where large volumes of water have been used. For example, Le Noir *et al*. [70] described the extraction of 17-β-estradiol from 2 L of effluent water taken from a waste-water treatment plant. Other interesting examples have been reported by Zhu *et al*. [37], who extracted four different pesticides from 1 L of river and tap water, and by our group [71], which reported on the extraction of naphthalene sulphonates from 1 L of river water. In other studies volumes of up to 100 mL of water have also extracted, such as in Sambe *et al*. [46], who reported on the extraction of triazines from river water, and our own study [25], in which we extracted carbamazepine from effluent water taken from a waste-water treatment plant. In all of these cases, the use of MIPs not only enabled a selective extraction of the analytes of interest but also an important preconcentration of the sample.

An indirect conclusion that can be drawn from the aforementioned examples is that the application of MIPs to water-based matrices is becoming feasible. Table 3 summarises some other studies that support the use of water as the matrix for extracting the analytes. However, retention to the MIP of the analytes of interest when they are in aqueous matrices is highly dependant upon both

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Table 3. Applications of MISPE to environmental samples.

Template	Funct. Mon.	X-Linker	Analyte	Pol. Type	Matrix	Anal. Method	On/Off-line	Ref.
17-β-estradiol	Acrylamide	--	17-β-estradiol	Traditional	Sewage water	LC	Off-line	[70]
Imidacloprid	MAA	EGDMA	Imidacloprid	Traditional	Water, Soil	LC-UV	Off-line	[84]
Ciprofloxacine	MAA	EGDMA	Enoxacin, Norfloxacin, Danofloxacin, Enrofloxacin	Precipitation	Soil	LC-UV	Off-line	[38]
Pinacolyl methyl-phosphonic acid	MAA	EGDMA, TRIM	Pinacolyl methyl-phosphonic, Ehylmethyl-phosphonic acid	Traditional	Soil	LC-MS	Off-line	[90]
Propazine	MAA	EGDMA	Simazine, Atrazine, Propazine, Terbutilazine	Precipitation	Water (river)	LC-UV	Off-line	[73]
Thiabendazole	MAA	EGDMA	Benzimidazolic compunds	Precipitation	Water (tap, river, well)	LC-UV	On/Off-line	[39]
Ethynylestradiol	MAA	EGDMA	Ethynylestradiol	Traditional	Water (river)	LC-UV	Off-line	[69]
Cyclobarbital	4-VPy	EGDMA	Phenobarbital, Cyclobarbital, Amobarbital, Phenytoin	Multi-step	Water (river)	LC-MS	On-line	[45]
Diclofenac	2-VPy	EGDMA	Diclofenac	Traditional	Water (river, sewage)	LC-UV	Off-line	[68]
2,4-dinitrophenol	4-VPy	EGDMA	2,4-dinitrophenol	Traditional	Water (tap, river)	FI-CL	On-line	[28]
Atrazine, Ametrine, Irgarol	TFMAA, MAA, 4-VPy	EGDMA	Symetrin, Ametryn, Prometrin,	Multi-step	Water (river)	LC-UV	On-line	[46]
Pentachloro-phenol	APTES	TEOS	Pentachlorophenol	Silica particles	Water (lake, river, sewage)	LC-UV	On-line	[83]
Bisphenol A	MAA, 4-VPy		Bisphenol A	Traditional	Water (bottled), Milk	LC-MS	Off-line	[19]

the polarity of the substance and the composition of the MIP. As Claude *et. al*. [26] and Fan *et al*. [72] reported, the MIP mainly retains those analytes through non-selective interactions such as electrostatic or hydrophobic interactions. In any case, once the MIP is loaded, selectivity is achieved by fine tuning a suitable clean-up solvent that is able to disrupt all those compounds which cannot be selectively retained on the MIP without disrupting the compounds retained through selective interactions [19, 38].

Nevertheless, as stated previously, the generally low capacity of MIPs means that in some cases directly preconcentrating the compounds of interest onto the MIP is not enough to reach the desired detection limits. In these cases, the most widely used strategy for increasing the volume that can be extracted with the MIP is a tandem system. In this case, a high-capacity commercially-available sorbent is used to preconcentrate the sample and, once this cartridge is extracted, this fraction is then percolated through the MIP to achieve the desired selectivity. This approach was chosen by Claude *et al*. [26] to extract tamoxifen and its main metabolite from a urine sample and, as Fig. 4 shows, the selectivity of the method is clearly improved if a MIP is used after extraction of the raw matrix with a commercial SPE sorbent, in this case Oasis® HLB. A similar example was reported by Carabías-Martínez *et al.* [73] regarding the extraction of metabolites of triazine compounds from river samples. In this case the authors used the commercial sorbent LiChrolut EN cartridge to preconcentrate the sample and the extract of this sorbent was then percolated through the MIP to achieve the desired selectivity.

Even so, there are some cases where concentration of the analytes is very low and, therefore, the use of highly sensitive detectors, such as mass spectrometry (MS) or MS-MS is required. For example, Anderson *et al*. [74] and Möller [75] used MS after a MISPE protocol to detect benzodiazepines in post-mortem hair samples and flame retardant agents in human urine, respectively. Other examples were reported by Harun *et al*. [76] and Demeestere *et al*. [57] who used MS-MS for detecting ketamine and norketamine in hair samples and antidepressants in environmental waters, respectively.

A clear advantage of using MIPs as sorbents for the LC-MS technique with electrospray ionisation is the decrease in the ion enhancement/suppression that is normally typical of this ionisation [56]. This occurs because the selectivity of the MIP enables a strong clean-up of the sample to be performed and, therefore, discards most of the organic matter present in the sample responsible for this ion enhancement/suppression. In some cases, the selectivity of the MIP is so high that it enables the analytes to be determined even after the MISPE protocol. For example our group [77] reported the direct detection by MS of the target analyte after the MISPE protocol without any previous chromatographic separation.

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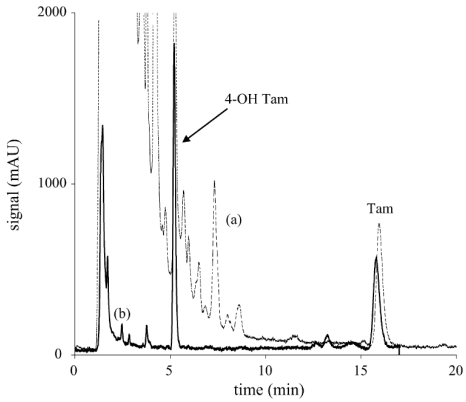

Figure 4. Chromatograms obtained after percolating human urine spiked with 0.125 mg L^{-1} of tamoxifen and 0.125 mg L^{-1} of 4-hydroxytamoxifen (a) through a commercial SPE sorbent and (b) after a tandem system using the commercial sorbent and the MIP [26] (reprinted with editor's permission).

Tables 1-3 include relevant examples of MISPE application in the most widely used samples over the last few years. As can be seen in Table 1, the most widely studied foodstuffs are vegetables [64, 78] rather than samples of animal origin and, the most widely studied compounds in vegetable samples are pesticides [21, 23]. Milk is the most widely studied sample of animal origin [19, 79, 80] and the most widely studied compounds in animal samples are antibiotics [29, 48, 59].

The most commonly used biological samples are plasma and urine (as summarised in Table 2) because these are the samples which contain the most important information for assessing the health of an individual. For example, Sun *et al*. reported on the synthesis of two very similar MIPs aimed at detecting fluoroquinolone antibiotics in either serum [81] or urine [82] samples. Apart from the cross-linking agent used, the synthetic conditions were the same for both polymers and, in both cases, the polymers obtained showed useful cross-selectivity for other compounds from the same family. The MIP used in serum samples enabled the detection of six different fluoroquinolones whereas the MIP used in urine samples enabled the extraction of up to nine different compounds. In both cases, the limits of detection for the fluoroquinolones were very similar when using LC/UV, the limits being 0.01 mg L^{-1} for serum samples and 0.1- 0.03 mg L^{-1} for urine samples.

The most commonly studied matrices from environmental samples are water and soil samples. Table 3 shows that water has been by far the most widely studied matrix and demonstrates that MIPs are being successfully used to extract compounds of interest from aqueous matrices. In this field, the most studied compounds are pesticides such herbicides [23, 37, 83] or insecticides [39, 84, 85]. The most commonly studied herbicide compounds are triazines. Studies reported in the bibliography show that all the MIPs developed for triazinic compounds displayed strong cross-selectivity for quite a large number of compounds that were structurally related to the template used. For example, Cacho *et al.* [21] reported using a MIP obtained under PP and following the semi-covalent approach to extract 5 different triazines from soil, corn and potato. The limits of detection when using LC-UV ranged between 0.4 – 1.1 µg Kg^{-1} for vegetables and 0.4 – 2.5 µg Kg^{-1} for soil. Carabias-Martínez *et al.* [73] reported another example of using MIP to extract triazines and their metabolites. In this case the authors applied the MIP to extract some triazines and their metabolites from river water samples and obtained a limit of detection between 0.1 – 0.03 µg L^{-1} using LC-UV. The MIP used was obtained under the PP protocol and the non-covalent approach and used the same template as Cacho *et al*.

In general and regardless of the matrix used, the data reported in bibliography show that there are several compounds which, due to their potential hazard to health, have been studied in many different matrices. This is the case of fluoroquinolones which are antibiotics which, in some cases, have been misused, so controlling them is of prime interest. These compounds have been extracted from biological, environmental or foodstuff samples and, in most cases, the MIPs obtained showed useful cross-selectivity. This is the case, for example, of Benito-Peña *et al.* [86], who synthesised a MIP using enrofloxacine as the template molecule and extracted four different quinolones from urine samples; or Caro *et al*., who reported on the extraction of enrofloxacine and ciprofloxacine from human urine and pig liver [24].

Some other particularly interesting papers on the synthesis of MIPs for targeting fluoroquinolones are those reported by Sun *et al.* [81, 82] and Yan *et al.* [87]. In these studies the authors used oxfloxacine as the template molecule and a mixture of MeOH:H_2O (9:1) as the porogen. This porogen is quite unusual since both of the solvent used are polar and protic solvent and the presence of such solvents during the synthesis of the MIPs is preferably avoided for not disrupting the proper interaction between the template molecule and the functional monomer. However, in all of these cases the MIPs obtained showed not only a good imprint effect but also good cross-selectivity for other fluoroquinolones.

Another family of compounds which has caught the attention of many different researchers over the last few years is that of herbicides and, in particular, triazines. These have been studied not only in environmental applications, but also in other kinds of matrices. Due to their high toxicity and widespread use, these compounds must be tightly controlled, and this is most effectively achieved by MIPs because of their high selectivity. Consequently, commercially-available MIPs already exist for the selective extraction of triazines. In this respect, a particularly interesting case was reported by Lara *et al.* [54], who used a commercial MIP for the selective extraction of atrazine and its three N-dealkylated metabolites from urine samples.

In all the papers reported in the bibliography, the most widely studied compounds for the selective extraction of triazines are atrazine and simazine. In all these cases the limits of detection were at the low µg Kg^{-1} when UV was used as the detection system. This fact proves, once again, that the high selectivity of MIPs enables the compounds of interest to be detected at concentration levels that would not have been obtained with conventional SPE sorbents unless more selective detectors, such as MS, were used.

4. Conclusions

Traditional polymerisation is still the most widely used synthetic protocol for the synthesis of MIPs, although over the last few years interest has grown in precipitation polymerisation as the technique of choice for overcoming the drawbacks of traditional polymerisation. Thus far, the most widely used approach for obtaining MIPs is the non-covalent approach using commercially-available functional monomers, although the use of self-designed functional monomers for the target analyte increases the affinity of the MIP towards the target molecule and, therefore, the possibility of obtaining cleaner extracts.

MIPs are mainly used for their selectivity rather than to preconcentrate the sample. Another interesting aspect to highlight is that, over the last few years, there has been a growing number of publications regarding the use of MIPs for performing selective extractions directly from water, thus overcoming the limitations that this matrix presented when MISPE was first developed.

Nevertheless, despite the large number of studies describing the synthesis of MIPs, imprinting large and highly functionalised molecules still remains a challenge.

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2. Experimental Part and Results

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2.1 Traditional Polymerisation for Molecularly Imprinted Polymers

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In this chapter we report on the synthesis of two MIPs using two highly polar compounds as template molecules and the feasibility of using the obtained polymers for the selective extraction of the target analytes from aqueous matrices.

The MIPs were synthesised using the Traditional Polymerisation (TP) protocol. The reasons for that were, on the one hand, its ability to obtain the imprinted materials that, as seen in the introduction, makes this approach the most widely-used nowadays and, on the other hand, because this is probably the best starting point for getting into the field of synthesising imprinted materials.

Once the MIPs were obtained, we were interested in assessing their ability to extract the target analytes from aqueous matrices and whether there was any difference in the extraction efficiency depending upon the template molecule used in the synthesis of the MIP.

To synthesise the MIPs, the template molecules used were two β-lactam antibiotics: amoxicillin (AMX) and cephalexine (CFX). Both of them are highly polar and functionalised compounds and, therefore, insoluble in the organic solvents normally used during the imprinting process. To overcome this drawback and to make them soluble in the solvent we were interested in, we derivatised them to their tetrabuthyl ammonium salts. This derivatisation enabled the template molecules to be soluble in ACN, the solvent used as the porogen in the polymerisation process. This derivatisation did not cause difficulties to the proper formation of active sites within the polymers due to the part of the molecule where this polymerisation was performed. The derivatisation process is not an uncommon procedure to make analytes soluble in the solvent of interest. For example, Urraca *et al*. followed this procedure to solubilise penicillin G in acetonitrile as its procaine salt [1].

For both MIPs, the functional monomers used were methacrylic acid. The interaction of this compound with either AMX or CFX is expected to be very similar since both AMX and CFX share almost the same functionalities, are in the same location and are very similar in size and shape.

As a cross-linking agent, ethylene glycol dimethacrylate (EGDMA) was the monomer of choice because of its markedly polar character compared to other cross-linking agents used in imprinting technology, such as divinyl benzene. The aim of using EGDMA as the cross-linking agent was to impair the polar characteristics of the polymer obtained. This is because the more polar characteristics the sorbent has the less affinity this polymer has for retaining the most hydrophobic compounds present in the sample, especially when extracting the target analyte directly from aqueous matrices. To this end, anything designed to decrease the retention of hydrophobic compounds on to the MIP is highly desirable. This is important because these compounds are retained through non-selective interactions and their total concentration is much higher than the concentration of the target analytes. When

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these compounds are retained on the MIP, the active sites on it can be hidden and so analytes of interest cannot be retained.

This feature was particularly desirable in the two studies presented in this chapter because target analytes were expected to be extracted from aqueous matrices in both of them. For the MIP imprinted using AMX, the sample assayed was human urine and, in the case of the MIP using CFX, the samples studied were human urine and river water samples.

As stated previously, the templates used in the synthesis of the MIPs were two β-lactamic antibiotics: AMX and CFX. However, these compounds belong to two different sub-groups of the β-lactamic antibiotics. AMX is a penicillin while CFX is a cephalosporin. The difference between these two classes is the 5-membered sulphured ring attached to the β-lactam characteristic for penicillins and the 6-membered sulphured ring characteristic for cephalosporines.

The main reason for selecting these compounds was because both AMX and CFX are highly functionalised compounds belonging to β-lactamic antibiotics. For these highly polar compounds, the synthesis of a MIP using such compounds as template molecules and the further development of a MISPE protocol for their selective extraction from highly complex and polar matrices, such as human urine and river water samples, represents a big challenge.

The other reason was because both of them belong to the antibiotic family and antibiotics are compounds widely used because of their high efficiency in the treatment of many human and animal diseases. However, in the latter case, the use of antibiotics has not only been exclusively used for treating diagnosed diseases but also as a prophylactic treatment [2] or, even worse, as growth promoters [3,4]. Therefore, the presence of antibiotic compounds in the samples used in the present study is not unexpected.

In any case, the abuse and misuse of antibiotics is dangerous because it can lead to the development of resistance to the targeted organisms (thus leading to a dramatic decrease in the efficiency of these antibiotics) and also the sensitivity of the organisms being treated. From this perspective, a more exhaustive control over those substances is needed if proper use is expected.

Although there are several papers describing the synthesis of MIPs as sorbents for the selective detection of antibiotics [5-7], in most cases these antibiotics belong to different families of compounds than the β-lactamic antibiotics. To the best of our knowledge, there were only two papers dealing with the preparation of MIPs using β-lactamic antibiotics when we synthesised these two MIPs. The first MIP was described by Lai *et al*. [8]. The authors used CFX as the template molecule and (2-(trifluoromethyl) acrylic acid (TFMAA)) as functional monomer to synthesise the polymer instead of methacrilic acid as in our case. Afterwards, the authors developed a MISPE protocol for the rapid screening of CFX in human plasma and serum and

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obtaining good results for the target analyte. The other MIP was described by Urraca *et al*. [1]. In this case, the authors applied the MIP in the selective extraction of penicillin derivatives from aqueous matrices. The most relevant aspect of the study - apart from the good results obtained with the synthesised MIP - was the use of a designed functional monomer according to the functionalities of the target molecule. The functional monomer was a urea-based monomer that had a strong interaction with the template used - the procaine salt of penicillin G in this case. The MIP obtained showed marked cross-selectivity for several penicillin G derivatives and enabled their extraction from tap and river water.

Since then, another MIP using AMX as the template molecule has been recently described and successfully applied in the determination of AMX in urine samples. This MIP was reported by Fowey *et al*. [9] and the authors used the same functional monomer and cross-linking agent. They also used the same ratio of all the components involved in the polymerisation process, as we did in the synthesis of our polymer. However, in this case the authors did not detect AMX by liquid-chromatography and UV detection after a MISPE protocol - as we did - but used flow-injection analysis and chemiluminiescence instead to obtain good recoveries and detection limits.

Despite the fact that these matrices are highly complex in nature, the use of the MIP as a sorbent in a SPE protocol enabled a selective extraction of the target analytes.

The results obtained in these studies were published in Journal of Separation Science. The notation for the study involving the MIP obtained using AMX as the template molecule is J. Sep. Sci., 2008, 31, 2868-2874, whereas the notation for the MIP obtained using CFX as the template molecule is J. Sep. Sci., 2009, 32, 3319-3326. It is also worth noting that the latter MIP study was published in a special volume of this journal published in 2009 that was dedicated to imprinting technology.

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2.1.1 Selective solid-phase extraction of amoxicillin and cephalexin from urine samples using a molecularly imprinted polymer

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Original Paper

Selective solid-phase extraction of amoxicillin and cephalexin from urine samples using a molecularly imprinted polymer

In this paper we describe, for the first time, a molecularly imprinted polymer (MIP) for the antibiotic amoxicillin (AMX), synthesised by a noncovalent molecular imprinting approach and used to extract AMX selectively from urine samples. The MIP was applied as a molecularly selective sorbent in molecularly imprinted SPE (MISPE) in an off-line mode, where it showed useful cross-selectivity for a structurally related antibiotic, cephalexin (CPX). By using a MISPE protocol, the MIP was able to selectively extract both AMX and CFX from 5 mL of water spiked with 10 mg/ L with recoveries of 75 and 78% for AMX and CFX, respectively. When applied to real samples (urine) at clinically relevant concentrations, recoveries from 2 mL of human urine spiked with 20 mg/L decreased slightly to 65 and 63% for AMX and CFX, respectively. To demonstrate further the selectivity of the MIP obtained, a comparison with commercially available SPE cartridges was performed. Improvements in the retention of both AMX and CFX on the MIP were obtained relative to the commercially available cartridges, and the MISPE extracts were considerably cleaner, due to molecularly selective analyte binding by the MIP.

Keywords: Amoxicillin / Cephalexin / Molecularly imprinted polymer / Solid-phase extraction / Urine samples

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1 Introduction

Antibiotics are used widely in human and animal treatments against diseases, but in the case of animals these compounds are sometimes also used in prophylactic treatments and even as growth promoters. The misuse and abuse of antibiotics is therefore becoming a great concern because drug resistance in the targeted micro-organisms or sensitivity in the organisms being treated may arise. There are several reports regarding the build-up of resistance to antibiotics by micro-organisms [1, 2], highlighting a decline in their efficiency unless a more rational and controlled use of such compounds is established.

In order to quantify the presence of antibiotics in different matrices, many new analytical methods have been developed [3–6]. These methods are, in most cases, based on LC in conjunction with either MS or UV detection. Several methods have been proposed for the determination of antibiotics in liquid samples, such as the environmental waters, with SPE being the technique used most frequently [7–9]. However, the lack of selectivity of the common SPE sorbents makes the extraction of these compounds from complex matrices rather difficult. Regarding selectivity issues, molecularly imprinted polymers (MIPs) have arisen as a powerful new force in SPE. As is well known [10–12], MIPs are tailor-made polymeric materials that are able to bind to target analytes present in many different sample matrices, and thus have been used in many different fields. Regarding analytical applications, MIPs have been used primarily as sorbents in SPE, in the emerging technique of molecularly imprinted SPE (MISPE) [13–15]. In many cases, MIPs are relatively easy and inexpensive to prepare and can be reused many times without losses in the performance.

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Abbreviations: **AIBN**, 2,2'-azobisisobutyronitrile, **AMX**, amoxicillin; **CPH**, cephazolin; **CPX**, cephalexin; **CPZ**, cephoperazone; **EGDMA**, ethylene glycol dimethacrylate; **MAA**, methacrylic acid; **MeOH**, methanol; **MIP**, molecularly imprinted polymer; **MISPE**, molecularly imprinted SPE; **NIP**, nonimprinted polymer; **TBA**, tetrabutylammonium; **TBT**, *tert*-butanol

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Moreover, they can withstand the use of organic solvents and extremes of pH. MIPs are polymeric sorbents normally synthesised in the presence of the analyte of interest; the analyte functions as a template in a templatedirected synthesis. In many cases, the resultant polymer is able to retain not only the target molecule but also structurally related compounds, a phenomenon known as cross-reactivity or cross-selectivity [16].

Although there are some reports describing the use of the MISPE protocols for the selective extraction of antibiotics such as tetracyclines and quinolones [17, 18], to date there are very few reports regarding the selective extraction of β-lactam antibiotics from real samples using MIPs. Kempe and coworkers [19] reported on the quantification of penicillin G (a β-lactam antibiotic) in commercial formulations and also worked to establish the optimum polymer synthesis conditions to deliver a MIP with the best recognition properties for penicillin G [20]. However, in these particular examples the ability of the resultant polymer to selectively extract the target analyte from a mixture was demonstrated by means of a competitive binding assay rather than MISPE. More recently, Sellergren and coworkers [21, 22] synthesised a MIP using penicillin G as a template molecule, and specially designed and synthesised functional monomers in a stoichiometric ratio with the template. The selectivity of the resultant polymer was evaluated by using an optimised MISPE protocol and eight β-lactam antibiotics, including amoxicillin (AMX). Although AMX was not the template molecule used during the synthesis of the polymer, it was one of the target antibiotics to be extracted from either tap or river water. When 50 mL of either tap or river water samples were spiked and analysed using a MISPE-LC protocol, the recoveries for AMX were lower than 50%, whilst the recoveries for the rest of the compounds were greater than 93% (with the exception of ampicillin where the recovery was ~75%), when the same volume and concentration of sample were analysed.

The lack of availability of MIPs for the selective extraction of β-lactam compounds arises from the chemical properties of such compounds. β-Lactam antibiotics are highly functionalised, polar compounds, thus their solubility in the solvents normally used in conventional synthesis protocols for MIPs is poor, therefore obtaining imprinted polymers using these protocols is very tricky [23].

Taking all these considerations into account, a MIP imprinted using AMX as a template is described in this paper. This MIP was obtained by polymerisation by using a noncovalent approach in an organic solvent, with the goal being to develop a robust imprinted material which could be applied to MISPE for the selective extraction of AMX from urine samples. Cross-selectivity of the AMX imprinted polymer was evaluated using other β-lactam antibiotics such as cephalexin (CPX), cephoperazone (CPZ) and cephazolin (CPH).

2 Experimental

2.1 Reagents and standards

The monomers and template used for the production of the MIP and the nonimprinted polymer (NIP) were methacrylic acid (MAA) (functional monomer), ethylene glycol dimethacrylate (EGDMA) (crosslinker) and AMX (template). All these chemicals were purchased from Aldrich (Steinheim, Germany). 2,2'-Azobisisobutyronitrile (AIBN), from Acros Organics (Geel, Belgium), was used as the free radical initiator. MAA was passed through an alumina column and then distilled under reduced pressure. EGDMA was washed consecutively with 10% aqueous sodium hydroxide (NaOH), water and brine, dried over $MgSO_4$, then filtered and distilled under reduced pressure. AIBN was recrystallised from methanol (MeOH). AMX was converted into its tetrabutylammonium (TBA) salt by suspending AMX (0.73 g) in MeOH, together with a small quantity of a drying agent ($MgSO_4$). Thereafter, 1 M TBA in MeOH (2 mL) was added dropwise to the suspension under continuous stirring (the milky suspension clarified once the TBA addition was complete) and left for a further 2 h. Themixture was filtered and the filtrate was concentrated *in vacuo* to give the desired product in the form of white crystals. Prior to use, the salt was rinsed several times with acetone.

For chromatographic analyses or MISPE experiments, the solvents used were acetic acid (HOAc), ACN, acetone, MeOH and 1-propanol, all from SDS (Peypin, France), *tert*-butanol (TBT) from Aldrich and THF from Probus (Badalona, Spain). Phosphoric acid (H_3PO_4) was from Merck (Darmstadt, Germany), and NaOH was from Prolabo (Fontenai S/Bois, France). Water was obtained from a Milli-Q purification system(Molsheim, France).

To evaluate the ability of the MIP to retain β-lactam compounds besides AMX three cephalosporins (CPX, CPH and CPZ) were used as test analytes. A stock solution of each compound in water/MeOH (90:10 v/v) at 1000 mg/L was prepared every week and stored in the freezer. Diluted solutions were prepared daily from this stock solution. All of these compounds were obtained from Aldrich; their chemical structures are presented in Fig. 1.

2.2 Synthesis of molecularly imprinted polymer

The polymerisation mixture was composed of the AMX–TBA salt (0.61 g, 1.01 mmol), MAA (0.35 g, 4.03 mmol), EGDMA (3.99 g, 20.1 mmol) and AIBN (0.073 g, 0.4 mmol); these components were dissolved in dry ACN (5.60 mL) in a 25 mL thick-walled, glass Kimax culture tube. Prior to polymerisation, the solution was sparged with oxygen-free nitrogen for 5 min (to remove oxygen) and sealed under a nitrogen environment. The tube was then left in an incubator set at 60º C for 48 h. Afterwards,

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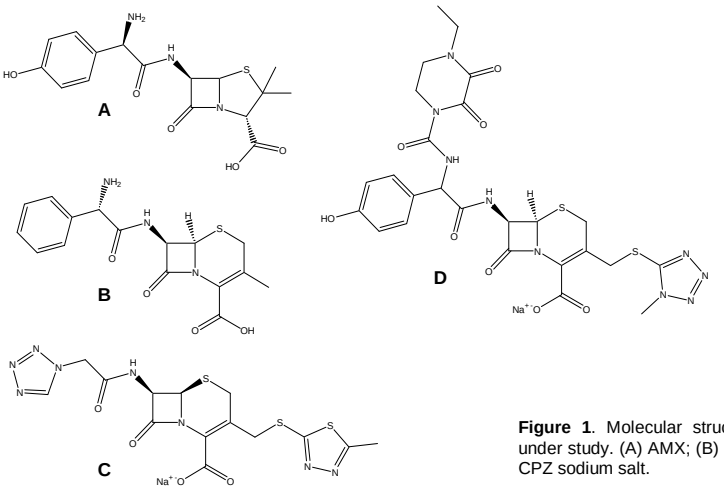

Figure 1. Molecular structures of the β-lactam antibiotics under study. (A) AMX; (B) CPX; (C) CPH sodium salt and (D) CPZ sodium salt.

the monolith obtained was crushed, mechanically ground and wet-sieved using acetone. The particles ranging in size from 25 to 38 µm were collected. Using the same synthetic protocol, but without the addition of the template molecule, a control polymer (NIP) was also prepared. The yields of particles suitable for use in the MISPE experiments and chromatographic evaluations (25– 38 µm size fraction) were 30% for the MIP and 8% for the NIP.

2.3 Equipment

Stainless steel LC columns (15 cm x 0.46 cm id), one for each polymer, were slurry-packed with ~1.5 g of the 25 – 38 µm particles using an air-driven, fluid pump (Alltech, Model 1666). The solvent used for slurrying and packing of the polymers was acetone. Chromatographic evaluation of the polymers was performed using an SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra- Physics, Mountain View, CA, USA).

For MISPE evaluations, 200 mg of each polymer (MIP and NIP) were packed into 6 mL polyethylene cartridges (Symta, Madrid, Spain). The cartridges were connected to an SPE manifold (Teknokroma, Barcelona, Spain), which was connected to a vacuum pump. The chromatographic equipment used to perform the analyses was an LC-10AD binary liquid chromatograph equipped with a DGU-14A degasser, an injection loop of 20 µL, a CTO-10A oven and an SPD-10A UV detector. All the instruments were from Shimadzu (Tokyo, Japan). The LC column used was a Kromasil 5 µm, 100 Å C_{18}, 250 mm x 4.6 mm from Teknokroma.

The MIP was compared to two commercially available SPE sorbents (polymers): Isolute® ENV+ (hydroxylated polystyrene–divinylbenzene copolymer, 200 mg), from IST (Mid Glamorgan, UK), and Strata™ SDB-L (styrene– divinylbenzene copolymer, 200 mg), from Phenomenex (Torrance, CA, USA).

2.4 Chromatographic conditions

For the chromatographic evaluations, 10 µL of a 1mM AMX solution in acidified water (pH ~3) and 2 µL of acetone (void marker) were injected, in turn, onto the MIP and NIP columns. Elution was carried out in isocratic mode, using ACN (containing 1% HOAc) as a mobile phase at a flow rate of 1 mL/min. The UV detector wavelength was set to 252 nm.

Chromatographic conditions for the separation of AMX, CPX, CPH and CPZ on the analytical column were stablished using a gradient of ACN and water (acidified with H_3PO_4 to pH ~3). The solvent gradient profile started with 10% ACN and reached 30% ACN within 4 min. From 4 to 6 min the ACN content increased to 50%, and from 6 to 8 min it increased again until 80%. From 8 to 12 min the ACN content was decreased to 10%. The total run time for each analysis was 12 min. The flow rate was 1 mL/min and the wavelength was set to 210 nm. The column oven temperature was set to 30ºC.

2.5 MISPE conditions

To ensure the efficient removal of the template from the MIP, the polymers in the MISPE cartridges were washed several times with 15 mL of acidified water (pH ~3) and 15 mL MeOH prior to use, until no AMX peak was observed in the eluted fractions of each solvent.

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MISPE experiments consisted of the conditioning of the cartridge by the percolation of 15 mL of acidified water (adjusted to pH ~3). The loading step consisted of the percolation of 2 mL of urine adjusted to pH ~3. After pH adjustment and before the MISPE step, urine was filtered in order to remove all precipitated material which could have blocked the cartridges.

Once the sample was percolated through the MIP, a clean-up step was performed using 5 mL of ACN in order to disrupt all the nonspecific interactions established between the MIP and all the compounds present in the sample.

MeOH (5 mL) was used as eluting solvent. The eluted fraction was dried under a stream of N_2 and reconstituted with 1 mL of acidified water/ACN (90:10 v/v) prior to injection on the liquid chromatograph. With Isolute ENV+ and Strata SDB-L, the same protocol was used.

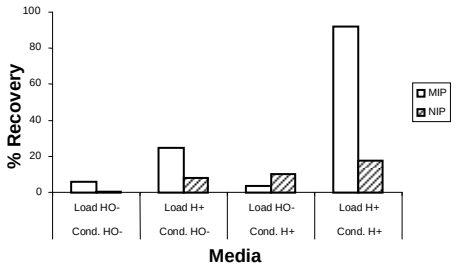

Figure 2. Mean recoveries (*n* = *3*) during sample loading under different cartridge and sample loading conditions. Acidic conditions were at pH ~3 and basic conditions at pH ~10. Sample: 5 mL of water spiked with 20 mg/L of AMX.

3 Results and discussion

3.1 Evaluation of the MIP

In order to probe themolecular recognition properties of the MIP, a chromatographic evaluation was performed. This involved the injection of 10 µL aliquots of 1 mM AMX in acidified water (pH ~3) onto the MIP and NIP LC columns, in turn. Under the chromatographic conditions specified in Section 2, the retention factors (*k'*) of AMX on the MIP (k'_{MIP}) and the NIP (k'_{NIP}) were calculated to be 22.61 and 1.67, respectively. When taking both the values together, the imprinting factor (IF = k'_{MIP}/k'_{NIP}) for the MIP was 13.56. This high value, when considered together with the pronounced peak-tailing (*i. e.* peak asymmetry) which was observed for AMX on the imprinted column, is already compelling evidence in support of a successful molecular imprinting outcome at the polymerisation stage.

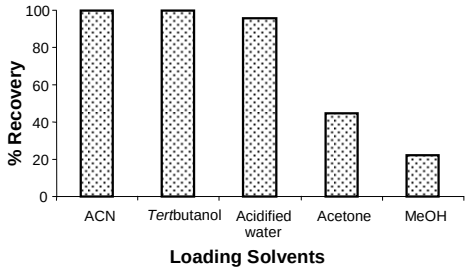

Figure 3. Comparative study of solvents used in the loading step. Five millilitres of each solvent was spiked with 20 mg/L of AMX.

3.2 MISPE

Once the SPE cartridges were packed with 200 mg of the imprinted polymer, a study of the conditioning of the cartridge and the pH of the sample was performed. In order to do this, 5 mL aliquots of water, spiked with 20 mg/L of AMX in either an acidic (pH ~3) or basic (pH ~10) environment were percolated through the cartridges, which had been activated previously with 15 mL of water in either acidic (pH ~3) or basic conditions (pH ~10). This involved four different trials. The eluted fractions were analysed and the AMX recovery calculated in order to determine the optimal MISPE conditions. As can be seen in Fig. 2, the optimal conditions were found to be when the cartridge was activated in an acid medium and the sample was present in a similar medium (both at pH ~3), since when the sample was percolated in basic media most of the AMX present in the sample was eluted directly in the loading step.

Therefore, in all subsequent MISPE experiments the sample was adjusted to pH ~3 and the cartridge activated by percolation of 15 mL of water (adjusted to pH ~3 with H_3PO_4) prior to any analyses.

In order to investigate the possibility of sample loading in different media, other solvents were tested as loading solvents. Several 5 mL solutions, based on different solvents spiked with AMX at 20 mg/L, were percolated through the MIP. As can be seen in Fig. 3, the solvents which gave the highest AMX recoveries were ACN, TBT and water (pH ~3), with water being the solvent of choice in subsequent work since urine is an aqueous sample.

The most effective eluting solvent found was MeOH; 5 mL of MeOH was sufficient to completely strip the AMX retained on the cartridge. When other commonly used solvents, such as acetone, THF or 1-propanol, were applied to the elution step, volumes greater than 5 mL were required. MeOH therefore disrupts the interactions between AMX and the MIP in a very efficient way, which is in accordance with the results shown in Fig. 3 (where

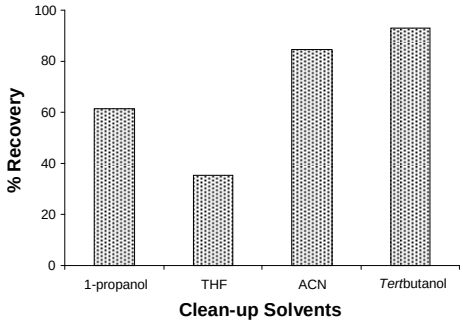

Figure 4. AMX recoveries following percolation of 5 mL of acidified water (pH ~3) spiked at 20 mg/L with AMX andapplication of 5 mL of various solvents in the clean-up step.

very low recoveries of AMX were obtained when the sample was loaded in MeOH).

Although MeOH was selected as the eluting solvent, prior to the quantification of AMX on the LC system it was necessary to evaporate and reconstitute the sample in 1 mL of acidified water/ACN (90:10 v/v) because when the sample in MeOH was injected directly onto the chromatographic system, peak splitting of AMX was observed. At the same time, this step also enabled the LOD of the analytical method to be lowered since the analyte was concentrated further by a factor of five.

When aqueous samples are loaded onto a typical polymeric SPE cartridge, many nonselective interactions, which are predominantly hydrophobic in nature, are established between the cartridge and the compounds in the sample. Since urine is an aqueous sample, many such interactions are expected to occur. In order to elute compounds from the cartridge that were bound by nonselective interactions, a clean-up step was included; this also enabled a cleaner chromatogram to be obtained.

After percolation of 5 mL of the AMX-containing acidified water sample (pH ~3) through the MIP cartridge, a clean-up step using 5 mL of washing solvent was performed. The selection of the solvent was done by taking into account the fact that other compounds present in the urine sample are quite polar and may interfere in the determination of AMX, so the solvents selected were 1-propanol, THF, ACN and TBT. As shown Fig. 4, ACN and TBT were found to be the best solvents to use in this step, with ACN eventually being the solvent of choice. In order to demonstrate the usefulness of the washing step, not only in disrupting nonselective interactions between the MIP and other compounds in the sample but also in highlighting the molecular recognition power of the MIP, a comparative study between the MIP and the NIP was carried out. To this end, different trials using 5 mL of acidified water containing 20 mg/L of AMX were percolated through the MIP and the NIP, respectively. In all cases, the percentage of AMX broken-through during the loading step was a10% for the MIP and around 60% for the NIP.

After washing the cartridges with 5 mL of ACN and eluting with 5 mL of MeOH, the recoveries of AMX were 77% (n = 3 and RSD = 4.2%) for the MIP and 18% (n = 3 and RSD = 6.3%) for the NIP. These experiments underline further the profound differences between the MIP and the NIP that were first brought to light by the chromatographic evaluation.

Once the MISPE protocol had been established in its entirety, the molecular selectivity of the MIP was studied by using three different b-lactam antibiotics. The antibiotics used for this purpose were CPX, CPH and CPZ, all of which are representatives of the cephalosporine family. These compounds were selected because they are all β-lactams and are therefore related structurally to AMX (Fig. 1).

After percolation of 5 mL of acidified water spiked with 10 mg/L of each antibiotic through the MIP only AMX and CPX remained bound to the polymer, whereas CPH and CPZ were eluted during the washing step (as might be expected based upon an inspection of their rather similar chemical structures (Fig. 1)). The MIP therefore apparently shows useful cross-selectivity for CPX; the recoveries of AMX and CPX on the MIP were 75 and 78%, respectively (n = 3 and RSD = 6.4 and 8.2%, respectively).

3.3 Urine samples

Once the optimal MISPE conditions for the extraction of AMX from an aqueous matrix had been established, and since the MIP had showed cross-selectivity for CPX, we moved to the extraction of both AMX and CPX from the urine samples.

Since urine is a highly complex matrix, the direct quantification of AMX and CPX on the sample is sometimes difficult. However, when the MISPE protocol described herein was applied to this sample, quantification of both AMX and CPX was achieved easily. Urine samples were selected because, according to Hirsch *et al.* [24], between 80 and 90% of the intake of AMX is excreted as AMX, without any modification to its structure. This fact enables the MIP to be used potentially in the pharmacological surveillance of AMX.

Prior to analysis, urine was taken to pH ~3 by the addition of H_3PO_4 and then filtered through a 0.22 μm nylon membrane filter. When urine samples were analysed a decrease in the volume of the sample to be extracted was required in order to avoid losses in the loading step. This change was necessary because urine is an extremely complex sample matrix and many compounds compete for binding to the polymer, leading to relativity lower recoveries of AMX. More specifically,

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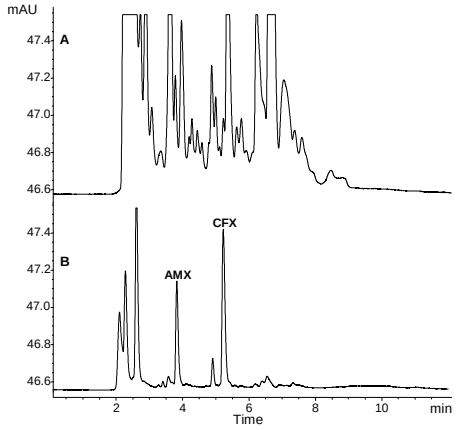

Figure 5. Comparison of chromatograms of: (A) direct injection of human urine sample; (B) the extract of 2 mL of human urine spiked with 20 mg/L after MISPE protocol.

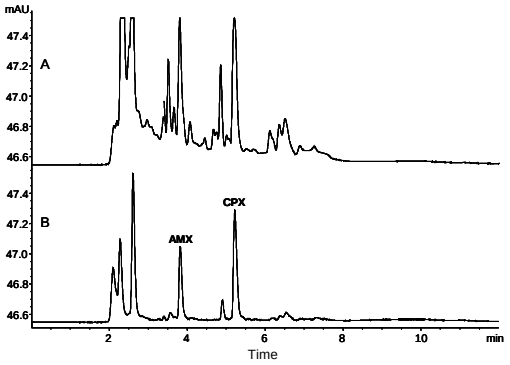

Figure 6. Chromatogram obtained after SPE of 2 mL of urine sample spiked with 20 mg/L of each compound using Isolute cartridge (A) and MIP (B).

instead of percolating 5 mL of sample through the cartridge, 2 mL of a urine sample was extracted. This lower volume led to reduced losses in the loading step; recoveries for AMX and CPX when 2 mL of acidified urine spiked at 20 mg/L with each compound was percolated through the MIP were 65% (RDS = 8.7%) and 63% (RDS = 12.3%) for AMX and CPX, respectively, from three different replicates.

To validate the method, human urine from healthy volunteers was spiked with different concentrations of both AMX and CPX to set linearity, repeatability and reproducibility between days. For linearity, the concentration range was established between 5 and 50 mg/L for AMX and CPX, giving a determination coefficient (r^2) of over 0.98 in both cases. The LOD of the method was 2 mg/ L for both compounds, which easily meets the clinical requirements in terms of method sensitivity for AMX when AMX is present in human urine samples [25]. However, it is worth noting that yet lower LODs can be obtained readily by the implementation of an even more sensitive detector such as mass spectrometer detector, to enable the quantification of AMX in biological samples besides urine, for example.

Repeatability was measured based on three consecutive, same-day repetitions of 2 mL of urine spiked at 10 mg/L. This gave RSD (n = 3) values of 6.9 and 4.6% for AMX and CPX, respectively. In a reproducibility between days test, analysis of the same sample concentration on three separate days gave %RSD (n = 3) values for AMX and CPX of 9.4 and 8.7%, respectively.

When the sample was percolated through the MIP many polar compounds were also retained on the MIP CPX was achievable following the clean-up step, enabling an accurate quantification of both compounds. Figure 5 shows the LC-UV chromatogram arising from direct injection of a human urine sample (A) and of 2 mL of human urine spiked with 20 mg/L of AMX and CPX after (B) the MISPE protocol. Once again, this fact corroborates the earlier data which highlighted the ability of the MIP to selectively extract AMX and CPX from fully aqueous samples.

In order to demonstrate the advantages of the AMX MIP over other SPE sorbents, a comparative study involving two commercially available cartridges was carried out. To this end, a hydrophobic Strata SDB-L (styrene – divinylbenzene copolymer) cartridge and a polymeric Isolute cartridge (hydroxylated polystyrene – divinylbenzene copolymer) with hydrophilic characteristics were selected. All the trials were carried out using 2 mL samples of acidified urine spiked with 20 mg/L of each compound and following the same protocol of the MIP.

After percolation of the samples through the hydrophobic Strata SDB-L cartridge, low recoveries of both compounds were obtained. This was expected because the retention mechanism for this type of sorbent involves hydrophobic interactions; however, AMX and CPX are highly polar compounds and are thus not retained. When the sample was percolated through the hydrophilic Isolute cartridge, the recoveries of AMX and PX were considerably better than the recoveries obtained on the hydrophobic Strata cartridge. However, because there is no in-built molecular recognition feature at play, the selectivity of binding was poor and the extracts were noticeably dirtier than those obtained from the MIP. Figure 6 shows a comparison of the extracts obtained from an SPE protocol on Isolute (A) and a MISPE protocol on MIP (B); the MISPE extract is cleaner, because molecular selectivity has been imparted into the MIP sorbent through the molecular imprinting process.

4 Conclusions

A MIP for the β-lactam antibiotic AMX has been prepared successfully in spite of its polar character. The AMX MIP demonstrated useful cross-selectivity, in that it was able to extract not only AMX from aqueous matrices but also the structurally related compound CPX, with recoveries greater than 60% for both compounds when 2 mL of urine was analysed. AMX and CPX are very polar compounds and very difficult to extract from aqueous samples using commercially available SPE cartridges, as has been re-affirmed in the present study. However, the reliable and reproducible MISPE/LC-UV protocol developed based on the AMX MIP as sorbent was able to efficiently extract both compounds from 2 mL of human urine allowing for facile quantification of the analytes at clinically relevant concentrations because of the cleanliness of the chromatograms obtained.

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2.1.2 Molecularly imprinted solid-phase extraction of cephalexin from water-based matrices

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Original Paper

Molecularly imprinted solid-phase extraction of cephalexin from water-based matrices

In the present paper, we describe the synthesis of a cephalexin (CFX) molecularly imprinted polymer (MIP), the direct application of the MIP to SPE for the determination of CFX (which is a β-lactam antibiotic) in human urine and the use of the MIP in a tandem SPE system to determine CFX in river water. The molecularly imprinted polymers (MIP) showed cross-selectivity for amoxicillin (AMX; also a β-lactam antibiotic). This allowed both CFX and AMX to be quantified in acidified human urine, with recoveries of 78 and 60% for CFX and AMX, respectively, when the urine samples were spiked with CFX and AMX at 4 mg/L. These analyses were facile because the molecularly imprinted solid-phase extraction (MISPE) extracts were clear compared to the nonpurified samples. In order to increase the sample volume for river water analyses, a tandem SPE system incorporating a commercially available sorbent was implemented. With this set-up, CFX was determined with recoveries in excess of 50% when 200 mL of acidified river water samples spiked at 10 μg/L with CFX were percolated through the tandem system.

Keywords: Amoxicillin / Cephalexin / Molecularly imprinted polymer / River water samples / Urine samples

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1 Introduction

With the ever-increasing demands for the monitoring of pollutants at very low concentrations, regardless of the matrix that they are in, analytical chemists have to deal with highly complex matrices. Amongst the many pollutants causing most concern, pharmaceutical compounds deserve special attention since, in many cases, these compounds are not fully eliminated in wastewater treatment plants [1] and may still be present in trace amounts when this water is reused. A common sample pretreatment technique used to reach low detection limits is SPE. Due to its simplicity, cost-effectiveness and the possibility of tuning the properties of the sorbent, SPE is used extensively and research efforts to develop new sorbents are on-going [2, 3]. However, these efforts are not likely to lead to the development of sorbents suitable for the quantification of single compounds at low concentrations in complex matrices. Where available, immunosorbents are actually rather good candidates in this regard [4, 5]; however, for many varied reasons the application of immunosorbents to the determination of a given compound of interest is not always possible.

To overcome this drawback of immunosorbents, whilst keeping a high selectivity for a target analyte, a new trend in SPE sorbents has arisen over the last few years; the aim is to deliver a sorbent specially designed to retain a specific target analyte. These new sorbents, which enable molecularly selective extractions, are called MIPs [6, 7].

The use of MIPs for the determination of various compounds, such as pharmaceuticals [8–10] and other organic pollutants [11–13] amongst others, has already been described. Antibiotics are a widely used family of pharmaceutical compounds for which extraction and quantification of the compounds at low levels is very difficult, so methods for their selective extraction are particularly desirable. There are some references in the literature describing the use of MIPs for the selective retention and subsequent quantification of different antibiotics such as penicillin G in human urine by competitive assay means [14], amoxicillin in human urine

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Abbreviations: **AIBN**, 2,2'-azobisisobutyronitrile; **AMX**, amoxicillin; **CFX**, cephalexin; **CPZ**, cefoperazone; **EGDMA**, ethylene glycol dimethacrylate; **HOAc**, acetic acid; **MAA**, methacrylic acid; **MIP**, molecularly imprinted polymer; **MISPE**, molecularly imprinted solid-phase extraction; **NIP**, nonimprinted polymer; **Pen V**, penicillin V; **TBA**, tetrabutylammonium

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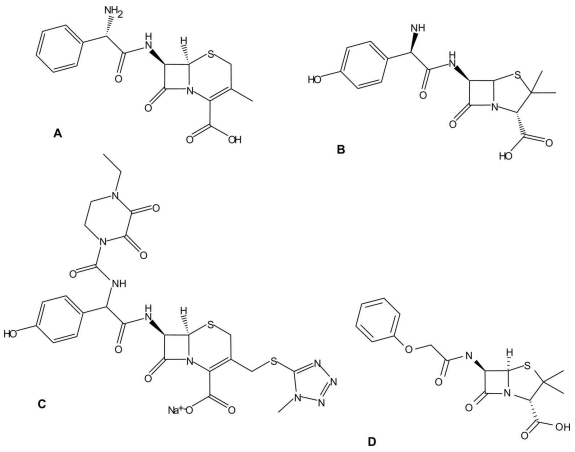

Figure 1. Chemical structures of the compounds used in the study: (A) cephalexin (CFX), (B) amoxicillin (AMX), (C) cephoperazone sodium salt (CPZ) and (D) penicillin V (Pen V).

using a MIP as a sorbent in an SPE protocol [15], and cephalexin (CFX) in human plasma and serum using molecularly imprinted SPE-pulsed elution (MISPE-PE) [16]. In this context, we decided to synthesise a MIP for CFX, using a modified synthetic protocol, and exploit the molecular recognition properties of the MIP in selective extractions of CFX from water-based matrices (urine and river water samples). These water-based matrices were selected for study due to the increasing concern over the presence of antibiotic compounds in environmental samples.

2 Experimental

2.1 Reagents and standards

The monomers used for the synthesis of the MIP and the nonimprinted polymer (NIP) polymers were methacrylic acid (MAA) (as functional monomer) and ethylene glycol dimethacrylate (EGDMA) (as cross-linking agent). For the MIP, CFX was used as template molecule. All reagents were obtained from Aldrich (Steinheim, Germany). Polymers were obtained *via* free radical polymerisation methods using 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium) as initiator.

All components used in the synthesis of the polymers were purified. To this end, MAA was passed through a neutral alumina column and then distilled under reduced pressure. EGDMA was washed consecutively with 10% NaOH, water and brine, then dried over $MgSO_4$, filtered and distilled under reduced pressure. AIBN was recrystallised from methanol.

The solvents used during the study were ACN, MeOH and acetic acid (HOAc) from SDS (Peypin, France). Phosphoric acid (H_3PO_4) was from Merck (Darmstadt, Germany), and sodium hydroxide (NaOH) was from Prolabo (Fontenai S/Bois, France). Water was obtained from a Milli-Q purification system (Molsheim, France).

To evaluate the ability of the MIP to bind selectively to CFX present in real samples, two penicillins (amoxicillin (AMX) and penicillin V (Pen V)) and a cephalosporin (cephoperazone (CPZ)) were used as analytes in addition to CFX. All of these compounds were obtained from Aldrich (Steinheim, Germany). Stock solutions of all four compounds (1000 mg/L of each compound in water/methanol [90/10]) were prepared weekly and stored in the fridge; diluted solutions were daily prepared. The chemical structures of CFX and the three other analytes are presented in Fig. 1.

2.2 Synthesis of molecularly imprinted polymer

Due to solubility problems with the parent compound, CFX was converted into its tetrabutylammonium (TBA) salt by suspending 1 g of anhydrous CFX in methanol followed by the dropwise addition of 3 mL of a 1 M solution of TBA hydroxide in MeOH in the presence of anhydrous $MgSO_4$. After 2 h of stirring at room temperature, the $MgSO_4$ was removed by filtration and the solvent was evaporated *in vacuo*. The product was obtained as yellowish crystals.

To prepare the MIP, CFX-TBA salt (0.59 g, 1.01 mmol), MAA (0.35 g, 4.03 mmol) and EGDMA (3.99 g, 20.1 mmol) were dissolved in 5.6 mL of dry ACN in a 25 mL thickwalled glass Kimax culture tube. Afterwards, AIBN (0.073 g, 1 mol% w. r. t. polymerisable double bonds) was dissolved in the solution and the solution deoxygenated for 5 min using oxygen-free nitrogen. The monomers were then left to polymerise for 48 h in an incubator set at 60ºC. Once the polymerisation was complete, the monolith obtained was ground, sieved and

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the fraction ranging from 25 to 38 µm collected, overnight Soxhlet extracted in MeOH and dried in readiness for the MISPE analysis. A scheme of the molecular imprinting procedure is shown in Fig. 2. In a similar fashion, but without the addition of the template molecule, a control (nonimprinted) polymer was obtained. The yields of the polymer particles in the size range 25–38 µm were 32% for the MIP and 8% for the NIP.

2.3 Equipment

To evaluate the polymers chromatographically, two stainless steel LC columns (5 cm x 0.46 cm id) were slurry-packed with ~500 mg of the 25–38 µm particles using an air-driven fluid pump (Alltech, Model 1666) with acetone as the slurrying and packing solvent. The MIP and the NIP columns were coupled, in turn, to an SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics,Mountain-View, CA, USA).

Two empty 6 mL polyethylene SPE cartridges (Symta, Madrid, Spain) were packed with 500 mg of either the MIP or NIP. The cartridges were connected to an SPE manifold (Teknokroma, Barcelona, Spain) which was connected to a vacuum pump.

MISPE extracts were analysed by LC using a LC-10AD binary liquid chromatograph equipped with a DGU-14A degasser, a 20 µL sample loop, a CTO-10A column oven and a SPD-10A UV detector from Shimadzu Corporation (Tokyo, Japan). The column fitted in the LC system was a Kromasil 5 µm, 100 Å C_{18}, 250 x 4.6 mm from Teknokroma.

To increase the volume of the sample being analysed, a tandem system using a commercially available sorbent in combination with the MIP was developed. The commercially available sorbents used were: Oasis® HLB (500 mg, 12 mL, *N*-vinylpyrrolidone-divinylbenzene copolymer) from Waters Corp. (Milford, MA, USA), Isolute® ENV+ (200 mg, 6 mL, styrene-divinylbenzene copolymer modified with hydroxyl groups) from IST (Mid Glamorgan, UK) and Bond Elut® CBA (500 mg, 3 mL, weak cation exchanger), from Varian (Middelburg, The Netherlands).

2.4 Chromatographic conditions

To evaluate the chromatographic properties of the MIP and NIP polymers, 10 µL of a 1 mM CFX solution in acidified water (acidified to pH ~3 with H_3PO_4) and 2 µlL of acetone (used as void marker) were injected onto both columns, in turn, using ACN (containing 1% v/v HOAc) as mobile phase at a flow-rate of 1 mL/min. Detection was carried out using a UV detection system monitoring at 252 nm.

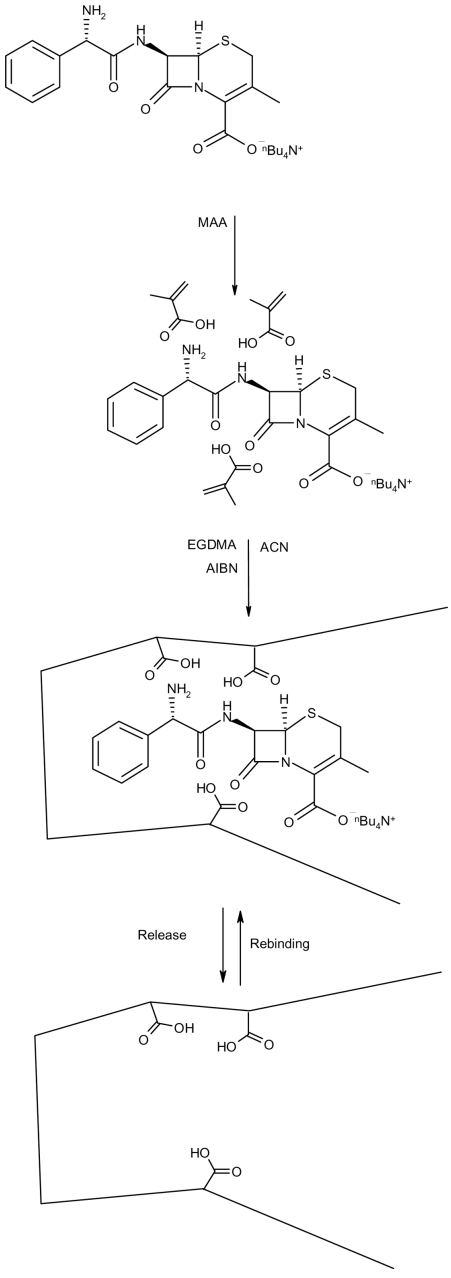

Figure 2. Schematic representation of the synthesis of the MIP.

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To assess the cross-selectivity of the MIP, the compounds used in this study were CFX, AMX, Pen V and CPZ. To separate these compounds, the chromatographic conditions involved a gradient of ACN and water (adjusted to pH ~3 with H_3PO_4) at a flow-rate of 1 mL/min and a UV detection wavelength of 210 nm. The gradient started with 10% of ACN and rose to 30% ACN in 4 min. After 6 min, the ACN content was 50% and was increased again to 80% from 6 to 8 min. From 8 to 12 min the ACN content was decreased to 10%. The column was maintained at 30ºC throughout.

2.5 MISPE conditions

Since the aim was to apply the MIP to human urine and river water samples, two different SPE protocols were established to deal with the fact that the matrices are very different, not least of all in terms of the typical concentration of CFX found in each matrix.

For urine samples, 2 mL of human urine (adjusted to pH ~3 with H_3PO_4) was filtered through a 0.22 µm pore size nylon membrane prior to percolation through the MIP. In this case, the clean-up step involved the percolation of 5 mL of ACN and 3 mL of acidified water (adjusted to pH ~3 with H_3PO_4), before elution of the bound analytes from the cartridge with 3 mL of MeOH (containing 1% v/v HOAc). The MeOH used to elute the analytes from the MIP was evaporated to dryness under a stream of N_2, reconstituted with 1 mL of acidified water (adjusted to pH ~3 with H_3PO_4) and quantified by LC.

In the case of river water samples, since the concentration of CFX in this type of sample was expected to be much lower than the CFX concentration in urine samples, but also because the MIP could only be used to extract relatively low volumes of river water samples, when analysing these samples a tandemsystem based on Oasis HLB-MIP was used. In this respect, 200 mL of water was acidified (adjusted to pH ~3 with H_3PO_4) and filtered through a 0.22 µm pore size nylon membrane prior to percolation through an Oasis HLB cartridge. Afterwards, the Oasis HLB cartridge was eluted with 10 mL of ACN (containing 5% v/v of HOAc) and this fraction was percolated immediately through the MIP. Finally, the MIP was eluted using 3 mL of MeOH (containing 1% v/v of HAOc). The eluate from this step was evaporated to dryness under a stream of N_2 and reconstituted with 1 mL of acidified water (adjusted to pH ~3 with H_3PO_4) prior to quantification by LC.

3 Results and discussion

3.1 Evaluation of the MIP

To investigate the affinity of the MIP for CFX, a chromatographic evaluation was carried out. To this end, two different experiments at different concentrations of CFX (1 mM and 10 mM) were carried out. Firstly, 10 µL of a 1 mM solution of CFX in acidified water (adjusted to pH ~3 with H_3PO_4) was injected onto the MIP and NIP columns in turn. Under the chromatographic conditions specified in the experimental section, the retention factors (k') for CFX were 33.4 and 1.5 for the MIP and NIP, respectively, which gave an imprinting factor (IF = k'_{MIP}/k'_{NIP}) of 21.7. Secondly, when a 10 mM solution of CFX was used instead of a 1 mM solution, the k' for CFX were 19.6 and 1.33 for the MIP and the NIP, respectively, giving an IF under these conditions of 14.7.

Overall, evidence in support of a successful imprinting outcome can be taken from both the high IF values measured and the broad, asymmetrical peaks in the LC which are typical for imprinted stationary phases.

3.2 MISPE

In order to probe the cross-selectivity of the MIP for structures closely related to the template molecule, we chose three β-lactamic antibiotics; a cephalosporin (cephoperazone (CPZ)) and two penicillins (amoxicillin (AMX) and penicillin V (Pen V)). Not only do these compounds share common structural features (Fig. 1), but they are also found in the same samples as CFX and at similar concentrations. Once an LC gradient for separating CFX, AMX, CPZ and PenV was established, the linearity of themethod between 0.5 and 50 mg/L was studied to enable the quantification of the recoveries in each step of the MISPE protocol and quantification of the analytes in real samples.

To perform the MISPE protocol, MeOH, ACN, acidified water (adjusted to pH ~3 with H_3PO_4) and basified water (adjusted to pH ~10 with NaOH) were tested as loading solvents. Ten microlitres of each solvent spiked with 2 mg/L of each antibiotic were percolated through the MIP; the recoveries for CFX, AMX, CPZ and Pen V when using MeOH as the loading solvent were 23, 12, 1 and 1%, respectively, whereas when using basified water (adjusted to pH ~10 with NaOH) the recoveries of the analytes were 25, 9, 5 and 6%, respectively. When using either ACN or acidified water as loading solvents, recoveries for CFX and AMX were >99% for both solvents. However, when using acidified water, CPZ and Pen V were also quantitatively retained (>99%) on the cartridge; by comparison, when using ACN as loading solvent the recoveries for these analytes were 22 and 27%, respectively. Therefore, acidified water was selected eventually as the preferred loading solvent since the MIP was intended to be applied to extractions from aqueous samples.

Since MeOH turned out to be the least effective solvent for loading of the MIP, we decided to use this solvent in the elution step. However, when using MeOH alone as the elution solvent, volumes over 10 mL were required to elute completely all the CFX retained on the

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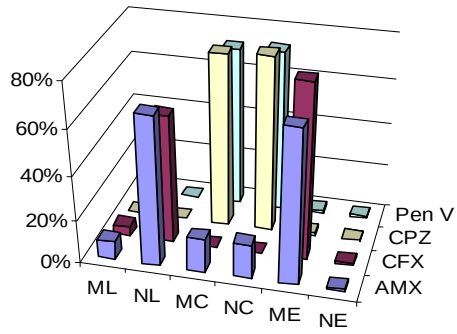

Figure 3. Comparison of MIP (M) vs. NIP (N) for the same SPE protocol. For loading (L) and cleaning (C) the values reported refer to losses in each step, whereas for the eluting (E) step the values are referred to recoveries. Sorbents loaded with 10 mL of acidified water spiked at 2 mg/L with each β-lactam, cleaned using 5 mL of ACN and eluted using 3 mL of MeOH (1% HOAc).

MIP, whereas the remainder of the compounds were eluted with lower volumes. To decrease the volume of elution solvent used and increase the preconcentration factor, several trials using HOAc as modifier for MeOH were tested; volumes of 2, 3 and 5 mL of MeOH containing 1, 5 and 10% of HOAc were studied. After different trials, the optimum volume and composition of the elution solvent was found to be 3 mL of MeOH spiked with 1% of HOAc, since when using this volume and composition of elution solvent over 99% of all the compounds were eluted from the MIP.

When attempting the analysis of real samples by MISPE, a clean-up step for removing nonselectively bound compounds from the sorbent prior to the elution step is highly desirable. From the data obtained for the loading step, ACN was selected as the clean-up solvent due to its organic nature which enables the disruption of the nonspecific interactions established in the MIP. The optimal volume was found to be 5 mL of ACN; after percolation through the MIP of 10 mL of acidified water spiked with 2 mg/L of each β-lactam all the compounds remained retained on the cartridge, whereas after a clean-up with 5 mL of ACN the recoveries for CPZ and Pen V were below 2% and the recoveries for CFX and AMX were 79 and 69%, respectively (n = 3).

Once the MISPE protocol was established in its entirety, a comparison between the MIP and the NIP was performed. Figure 3 summarises the results obtained when performing exactly the same SPE experiment using the MIP or the NIP as sorbent. As can be seen, the retention of the compounds under study is different in each step of the protocol. For instance, when 10 mL of acidified water (adjusted to pH ~3 with H_3PO_4) spiked with 2 mg/L of each β-lactamic antibiotic used in the study were loaded, in turn, to the MIP and the NIP cartridges, hardly any losses for any of the compounds was observed in the MIP whereas in the NIP losses of 57 and 68% for CFX and AMX, respectively, were observed.When introducing a clean-up step using 5 mL of ACN, CPZ and Pen V were eluted from both the MIP and NIP leading us to conclude that retention of these compounds was by nonspecific interactions rather than specific interactions as observed for CFX and AMX in the MIP. In this clean-up step, losses of 15 and 16% for AMX were found for the MIP and NIP. Once the cartridges were eluted with 3 mL of MeOH (containing 1% HOAc), recoveries after the complete SPE protocol were 79 and 69% for CFX and AMX, respectively, in the MIP whereas recoveries for all the compounds were below 1% whenthe NIP was used in the place of the MIP.

This data corroborate yet again not only the crossselectivity of the MIP towards AMX, but also the retention of CFX and AMX on the MIP is achieved through selective interactions rather than through nonspecific interactions.

Since the goal was to apply the MIP to extraction of both human urine and river water samples, a study to establish the maximum sample volume which could be percolated through the cartridge, whilst maintaining high analyte recoveries, was performed. Even though no clean-up step was performed in this study, only CFX and AMX were tested. CPZ and Pen V were omitted because both compounds were eluted during the clean-up step and this step was going to be included in the analysis of real samples. So, when 25 mL of acidified water was percolated through the MIP the recoveries of CFX and AMX were 99 and 97%, respectively (n = 3), whereas when higher sample volumes were used, for instance 50 mL, the recovery values fell to 60% for CFX and even lower for AMX.

To overcome the low capacity of the MIP in the case of river water samples where the concentration of all the compounds of interest is expected to be very low, a tandem extraction system comprising a commercially available sorbent and the MIP was developed [15]. The sorbents of choice for this tandem approach were Bond Elut CBA (weak cation exchanger), Isolute ENV+ (hydrophilic) and Oasis HLB (hydrophilic–lipophillic balanced). For all three commercial sorbents, 10 mL of acidified water (adjusted to pH ~3 with H_3PO_4) spiked with 6 mg/L of CFX and AMX were percolated through each cartridge and eluted using 5 mL of MeOH (containing 1% HOAc).

From the data obtained for the loading step, recoveries or both AMX and CFX were >98% for Oasis HLB, whereas recoveries for Isolute ENV+ were slightly lower and far lower in the case of Bond Elut. On this basis, both Bond Elut and Isolute ENV+ were not considered to be appropriate for use with the tandem system since yet higher losses of the analytes were expected when attempting the analysis of higher volumes of samples, as is normally required for river water samples.

Since the Oasis HLB cartridge was appropriate for use with the tandem system, the solvent used for elution of analytes from Oasis HLB ought to also be appropriate for use as the loading solvent for the MIP; the best candidate in this respect was ACN, so we first tested 5 mL of ACN as eluting solvent. However, 5 mL of ACN alone was not sufficient to elute completely CFX and AMX retained on the Oasis HLB, therefore trials using different volumes of ACN with different HOAc contents were attempted. More specifically, 100 mL of acidified water spiked with 4 mg/L of CFX and AMX were percolated through the Oasis HLB cartridge, and the analytes eluted with ACN containing HOAc. The optimal volume and composition of elution solvent for the Oasis HLB sorbent was found to be 10 mL of ACN containing 5% of HOAc, since this volume and composition was able to elute all CFX and AMX from the cartridge. When attempting to use 10 mL of ACN alone, there was still some CFX retained on the Oasis HLB. When increasing the HOAc content in ACN above 5%, the retention of both CFX and AMX was dramatically reduced when this fraction was subsequently percolated through the MIP cartridge. Once the optimal volume and composition were established for both eluting the Oasis HLB cartridge and loading of the MIP, we performed the complete MISPE protocol. The final volume of sample able to be percolated through the Oasis HLB cartridge was fixed at 200 mL of acidified water, and in this case, only CFX was quantified (recovery A90%). The reason for this is that even after percolation of 100 mL of acidified water, the recovery of AMX was only 42%, leading us to conclude that this tandem method with a CFX MIP does not allow for an effective extraction of AMX from aqueous samples.

Once the maximum volume of water able to be percolated through the Oasis HLB cartridge was established, we moved to an evaluation of the performance of the entire tandem system. To this end, 200 mL of acidified water (adjusted to pH ~3 with H_3PO_4) spiked with 0.03 mg/L of CFX was percolated through the Oasis HLB cartridge and eluted using 10 mL of ACN (containing 5% HOAc v/v). The eluate was percolated through the MIP sorbent and eluted using 3 mL of MeOH (containing 1% HAOc, v/v). After drying with N_2, the eluted fraction from the MIP was reconstituted in 1 mL of acidified water (adjusted to pH ~3 with H_3PO_4); the recovery of CFX was 60% (n = 3).

3.3 Real samples

3.3.1 Urine samples

Antibiotics are commonly prescribed pharmaceuticals which, once in the body, are excreted mainly *via* urine [17], so direct quantification of antibiotics from urine [18] without any tedious sample pretreatment is highly desirable. With this in mind, we wanted to quantify CFX and AMX in human urine and, since the concentration of the analytes is expected to be higher in urine than in river water samples, in this case it was not necessary to use the tandem.

After adjusting the urine sample to pH ~3, different sample volumes of 10, 5 and 2 mL of human urine spiked at 0.7, 1.4 and 3.5 mg/L with both CFX and AMX, respectively, were assayed. However, due to the large number of other compounds also present in the sample, the analysis was only feasible when using 2 mL of human urine since when the sample volume was increased the recoveries for the analytes decreased dramatically.

The clean-up step involved the percolation of 5 mL of ACN; however, since many ionic compounds are present in urine an extra step in the clean-up stage was included. This involved percolation of 3 mL of acidified water (adjusted to pH ~3 with H_3PO_4) [19], the aim being to decrease the amount of the polar compounds which are retained on the MIP through nonspecific interactions. Although this meant one extra step in the cleaning step, no further losses of any of the analytes were observed.

The MISPE cartridge was eluted with 3 mL of MeOH (containing 1% v/v HOAc) and the eluted fraction evaporated to dryness under a stream of N_2. After reconstitution of the sample with 1 mL of acidified water (adjusted to pH ~3 with H_3PO_4), recoveries from 2 mL of human urine spiked at 4 mg/L with CFX and AMX were 78 and 60%, respectively, with an RSD of 1.2% for CFX and 12% for AMX from three different replicates.

Figure 4 shows the comparison of two different chromatograms: Chromatogram A corresponds to the MISPE protocol of 2 mL of blank (nonspiked) human urine; chromatogram B corresponds to 2 mL of human urine spiked at 3.8 and 4.4 mg/L with CFX and AMX, respectively. As can be seen, a clean chromatogram was obtained after the MISPE protocol, enabling facile quantification of the compounds present in the sample.

When comparing the performance of the CFX MIP with a MIP reported previously by our group (which was synthesised using AMX as template molecule and which showed cross-selectivity for CFX) [15], the present MISPE protocol gave somewhat better recoveries for the analytes under study.

3.3.2 River water samples

The river water used in this study was from the Ter river. Since antibiotic concentrations in river water samples are expected to be far lower than their concentrations in urine samples, the tandem system described previously was used. In this case, and from results obtained with ultra pure water, AMX determination was not performed since the recoveries of AMX from 100 mL of ultra pure water were very low.

After the complete MISPE protocol (involving the tandem system) with 200 mL of acidified river water sample spiked at 10 µg/L with CFX, recoveries for this

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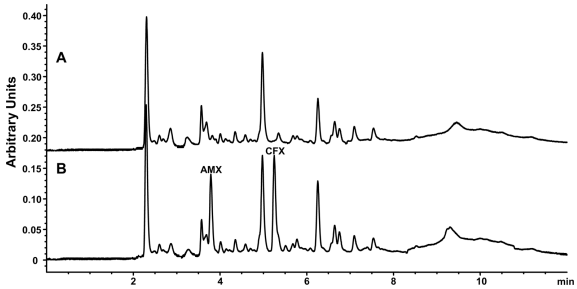

Figure 4. Chromatograms obtained from 2 mL of human urine after the MISPE protocol. A) spiked at 3.8 and 4.4 mg/L with AMX and CFX, respectively and B) blank of human urine.

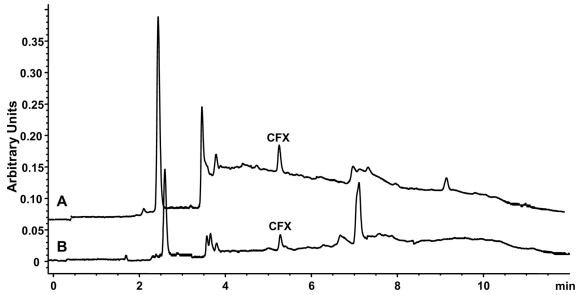

Figure 5. Comparison of chromatograms obtained after percolation of 200 mL of Ter river water spiked at 10 µg/L with CFX through A) Oasis HLB and B) tandem Oasis HLB-MIP.

compound were 53%. Figure 5 shows the comparison of the chromatograms obtained after the percolation of 200 mL of river water spiked at 10 µg/L with CFX through an Oasis-HLB cartridge (A) and after the tandem MISPE protocol using the commercial sorbent and the MIP (B). As expected, the target analyte was also retained on the commercial sorbent but, when comparing both chromatograms, the one obtained after the MISPE protocol has a flatter base line than that obtained using only the commercial sorbent meaning that this is a cleaner extract. As can be seen in Fig. 5, quantification of CFX was performed easily and, although the concentration of CFX spiked into the river water samples was rather high, lower CFX concentrations could have been determined by using a more sensitive detector, such as MS or MS/MS. If this were the case, the cleaner chromatogram obtained when using the tandem system would be a clear advantage for decreasing the ion suppression effect typical in ESI MS. Ion suppression can be a significant problem with MS when analysing dirty samples [20].

4 Concluding remarks

A MIP for CFX has been prepared which binds not only to CFX but which showed good cross-selectivity for AMX, a compound structurally related to CFX. A MISPE protocol was developed which enabled the selective extraction of both CFX and AMX from human urine and CFX from river water samples.

For human urine samples, quantification of both compounds was achieved easily despite the high polarity of both compounds and the fact that urine is an aqueous matrix. Recoveries of CFX and AMX from 2 mL of human urine spiked at ~4 mg/L were 78 and 60%, respectively.

For river water samples, due to the relatively low capacity exhibited by the MIP, a tandem extraction system using a combination of a commercially available sorbent with the MIP was developed. When using the tandem system, the entire SPE protocol led to a clear improvement in the analytical outcome compared to commercial sorbents; recoveries of CFX were over 50% when analysing 200 mL of river water sample.

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2.1.3 Discussion of Results

As can be seen in the previous section, two MIPs for two different β-lactamic antibiotics were successfully obtained by Traditional Polymerisation. In both cases, the MIPs enabled a selective extraction of not only the target analytes but also some closely related structures from water-based matrices.

These results not only demonstrate that TP is - as stated in the introduction - a very robust strategy in terms of imprintability but, more importantly, that the derivatisation process of the template molecule adopted in both cases turned out to be a very good option for solubilising the template molecule while not affecting the recognition properties of the final product obtained.

Another conclusion derived from the low mass of the suitable fraction of particles required for MISPE applications compared to the total mass of MIP produced is that, once again, it has been proven that most of the polymer obtained by Traditional Polymerisation must be discarded since cannot be used in the MISPE application. This is because of the long and tedious process of crushing, grinding and sieving the monolith obtained

In both cases, the MIPs were indeed imprinted as seen from the high values of the *Imprinting Factor* (IF) observed for them. This means that the target analyte is retained on the MIP by specific interactions. Therefore, both MIPs obtained showed enhanced selectivity towards the analyte used during the synthesis of the polymer.

As seen in the introduction, the selectivity of a MIP is based on a synergic effect. This selectivity arises from the proper match between the size of the target molecule, the size of the cavity left in the polymer and by the presence of complimentary binding sites to the target molecule within this cavity. So, when the target analyte is allocated within the polymeric cavity, its retention is enhanced by the presence of complimentary binding sites specially located in this cavity that strongly retain the analyte.

From this reasoning, all the molecules fitting the size of the cavity and having complimentary functionalities in the same position as the functional monomers are retained on the MIP.

With this consideration in mind, we were interested in checking the ability of the MIP to retain different molecules to the ones used as templates molecules but which share common structural features. When comparing the MIPs obtained, a very interesting result was the cross-selectivity displayed by all of the sorbents. The MIP synthesised using AMX as the template molecule displayed cross-selectivity towards CFX and vice versa. The MIP using CFX as the template molecule displayed cross-selectivity towards AMX. None of the polymers showed cross-selectivity towards the rest of the compounds under study.

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This cross-selectivity can be understood by having a close look at the structures of all of the compounds used during the different studies. Despite all of them being β-lactamic antibiotics, not all of them were either functionalised in the same position or had the same size. The fact that compounds of different sizes were not retained can be explained by the effect of steric impediments, which made it impossible to generate a proper fitting of the compound within the cavity whereas compounds of the same size were not selectively retained because did not have their functionalities in the right place. For example, Fig. 2 on the paper describing the synthesis and use of a MIP obtained using CFX as template molecule depicts the most likely interaction of CFX and the polymer. From that picture and considering the structure of all the compounds used in the two different studies, it is not difficult to visualise that both AMX and CFX have all the functionalities involved in the selective interaction in the same place whereas none of the rest of the compounds studied have those functionalities in that particular position. Therefore, the position of these functionalities in the right place has a dramatic effect. This result is supported by the fact that in all of the MIPs obtained using either AMX or CFX as template molecules, neither cephazoline, cephoperazone nor penicillin V were retained on the polymers despite having very similar structures to the template molecules.

This observation leads us to believe that the primary amino group present in both AMX and CFX plays a crucial role in the selective retention of both compounds on the MIPs studied. When this group is a secondary amino group, as is the case with cephoperazone, steric effects prevent the proper interaction in that position and, when that amino group is not present, as is the case with cephazoline or penicillin V, no retention of the compound is observed either.

All the above results were obtained from studies performed using ultra pure water but both MIPs synthesised were designed to be applied to human urine samples. Human urine was selected because we were interested in proving that the use of both MIPs was feasible in the selective extraction of β-lactamic compounds from highly complex matrices. Moreover, the use of the two MISPE protocols described would enormously facilitate the isolation and quantification of either AMX or CFX in urine samples since, according to Beograd *et al*. [10], most of the intake of antibiotics are excreted from the body almost unchanged *via* urine or faeces.

For the MIPs obtained by using AMX or CFX as template molecules, two millilitres of human urine sample were enough to quantify AMX and CFX. Although the volume used was rather low because of the complexity of the sample, the chromatogram obtained after the MISPE protocol was very clean and the compounds of interest could easily be detected and quantified using a UV detection system.

The low volume used in the two studies presented is not unusual in MISPE extractions. A reason for this is that the use of MIPs is more focused on performing a strong and effective clean up of the sample rather than achieving high preconcentration factors, as in the most common SPE treatments.

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Once the feasibility of extracting AMX and CFX from human urine samples was proven, we turned our attention to extracting β-lactamic antibiotics from river water samples. Due to the low concentration levels of the target analytes in river samples, a large volume of sample was required. Due to the low capacity exhibited by the MIP, a tandem system had to be used in order to reach the volume required. This system consisted of using a commercially available hydrophilic polymeric sorbent (Oasis HLB®) to retain the compounds of interest. Once this sorbent was eluted, the fraction was further percolated through the MIP to obtain the desired selectivity. However, since AMX is a more polar compound than CFX, AMX was omitted in the tandem system because some losses were already observed when percolating the volume of sample required through the commercial sorbent.

As reported previously, two different MIPs using β-lactamic antibiotics as template molecules were successfully synthesised and applied to human urine samples. The MIPs synthesised showed useful cross-selectivity for some other β-lactamic compounds but not for all of them. This result reflects the fact that only analytes with very similar sizes, shapes and functionalities can be retained on the MIP by selective interactions. When any of these prerequisites fail, the analytes are either not retained or washed away during the cartridge clean-up step.

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2.2 Comparison of two different polymerisation protocols for the synthesis of MIPs

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Once feasibility for obtaining imprinted materials using Traditional Polymerisation was proven, we moved on to study differences in the performances of MIPs obtained under different polymerisation protocols. To this end, we selected Traditional Polymerisation and Precipitation Polymerisation to assess the differences between these two synthetic protocols. Traditional Polymerisation was chosen because, as previously stated, this has been the most widely used synthetic approach for obtaining the MIPs used in MISPE applications. Precipitation Polymerisation was chosen because it is the polymerisation protocol that overcomes the main drawbacks of Traditional Polymerisation.

The main drawbacks of Traditional Polymerisation arise from the method of obtaining the range of useful particles for MISPE applications, once the MIP is synthesised. As stated in the introduction, the particles derived from this polymerisation protocol are irregular in size and shape. This irregularity makes it difficult to pack the cartridge properly and also diminishes the capacity of the sorbents, since the efficiency of the mass transfer of all the analytes on to the solid support is decreased.

In contrast, the product obtained for Precipitation Polymerisation is in the form of discrete spherical particles with a narrower dispersity than the particles derived from Traditional Polymerisation [1]. These particles are typically in the low micrometer range (2 – 5 µm) and, even though this is a rather small particle size for sorbents used in SPE protocols, there are some papers in which the use of these particle sizes have been applied successfully in MISPE applications [2,3]. In the case of the particles smaller than ~ 2 µm, they are only used in rebinding experiments because they would increase the backpressure of the system enormously in MISPE protocols.

The spherical shape of the particles obtained makes it much easier to pack the cartridges. This better packing of the cartridge makes the mass transfer of the analytes on to the sorbents more efficient which, in turn, increases the capacity of the sorbent.

In order to perform a proper comparison of the two kinds of polymerisation methods, we synthesised two different MIPs using the same monomer composition. Therefore, the MIPs were synthesised using the same functional monomer, the same cross-linking agent and the same ratio of those monomers compared to the template molecule. For both MIPs obtained, the template molecule was carbamazepine, an anticonvulsant drug normally used in epilepsy treatment.

Once the polymers were obtained, the main aim of the study was to assess the differences shown by the two MIPs in extracting carbamazepine from aqueous matrices. The differences observed should be a good way to compare the advantages and disadvantages of each kind of polymerisation protocol.

As in the previous chapter, the sample of choice was human urine because it is both aqueous and a highly complex matrix. Therefore, a direct comparison of the

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efficiency of the two MISPE protocols developed using either a MIP obtained under Traditional Polymerisation or Precipitation Polymerisation could easily be performed by using liquid chromatography and UV detection.

However, we decided to go one step further with the MIP obtained by Traditional Polymerisation by assessing how well it could extract the target analyte from effluent water from a wastewater treatment plant (WWTP). We were interested in this kind of sample because it is also an aqueous and highly complex matrix. Another reason for detecting CBZ in effluent water from a WWTP is because the presence of this compound has been reported both in effluent water and sludge from WWTPs [4,5], proving that this compound is not fully eliminated in these plants. Effluent water from WWTPs is mostly released into the environment and, therefore, all the compounds contained within it do so as well. This is an important issue since the fate and effects of these compounds is unknown and could represent a serious hazard to living beings, such as animals.

Another aspect we were interested in was the assessment of the cross-selectivity exhibited for the polymers synthesised. In the case of the MIP obtained under Traditional Polymerisation, the aim was to check the ability of the MIP when retaining similar molecules - in terms of size and functionalities - to the template molecule used and which are present in the same kind of samples as CBZ and at similar concentration levels.

All the results derived from the application of the MIP obtained by Traditional Polymerisation were published in Anal. Chim. Acta 597 (2007) 6-11. The results derived from the MIP obtained by Precipitation Polymerisation were published in J. Chromatogr. A, 1216 (2009) 2248-2253.

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2.2.1 Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater

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Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater

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Abstract

A molecularly imprinted polymer (MIP) designed to enable the selective extraction of carbamazepine (CBZ) from effluent wastewater and urine samples has been synthesised using a non-covalent molecular imprinting approach. The MIP was evaluated chromatographically in the first instance and its affinity for CBZ also confirmed by solid-phase extraction (SPE). The optimal conditions for SPE consisted of conditioning of the cartridge using acidified water purified from a Milli-Q system, loading of the sample under basic aqueous conditions, clean-up using acetonitrile and elution with methanol. The attractive molecular recognition properties of the MIP gave rise to good CBZ recoveries (80%) when 100 mL of effluent water spiked with 1 μg L^{-1} was percolated through the polymer. For urine samples, 2 mL samples spiked with 2.5 μg L^{-1} CBZ were extracted with a recovery of 65%. For urine, the linear range was 0.05–24 mg L^{-1}, the limit of detection was 25 μg L^{-1} and precision, expressed as relative standard deviation at 0.5 mg L^{-1} (n = 3), was 3.1% and 12.6% for repeatability and reproducibility between days, respectively.

Keywords: Molecularly imprinted polymer; Carbamazepine; Solid-phase extraction; Urine samples; Wastewater

1. Introduction

In many different fields, and for many different reasons, the identification and accurate determination of organic compounds is becoming of prime importance. Very often, the compounds are present at a very low concentration, which complicates their quantitation. Of the many different solutions proposed to enable the determination of organic compounds, one approach that has been particularly successful, due to its applicability to a broad spectrum of compounds present in many different sample matrices, has been the development of molecularly imprinted polymers (MIPs).

There are several distinct approaches to the synthesis of imprinted polymer particulates, including precipitation polymerisation [1,2], suspension polymerisation [3,4], swelling polymerisation [5] and grafting procedures [6]. However, the most commonly used technique is still polymerisation to give a solid monolith followed by grinding and sieving of the monolith [7]. This is the synthetic approach that has been adopted in the present study.

It is well-established that the main novelty of MIPs lies in their ability to bind to a particular compound of interest, or in some cases to bind a family of structurally-related compounds [8], present in a complex mixture. This feature is related directly to selectivity and, for analytical purposes, it is often highly desirable to develop a selective protocol to determine a single compound. This high selectivity is particularly desirable when a single compound has to be extracted from very complex matrices, making MIPs very attractive indeed as molecularly selective sorbents in solid-phase extraction (SPE) [9–11]. This is why the sample preparation technique of molecularly imprinted solid-phase extraction (MISPE) is gaining in popularity. MISPE has allowed isolation and quantification of many analytes, including fluorinated quinolones in biological samples [8–12]

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and anti-inflammatory drugs in environmental samples [7,13]. If, in addition to high selectivity, it is appreciated that imprinted polymers can be reused many times without significant losses in performance, and that they are relatively easy, inexpensive and quick to produce, then this makes them very attractive materials indeed as SPE sorbents. In passing, it is worthwhile noting that besides MISPE protocols, MIPs can also be applied in other fields, e.g., as sensors [14,15] and as catalysts [16,17].

The aim of the present study was to prepare a polymer imprinted with carbamazepine (CBZ), an anti-convulsant drug used widely in epilepsy treatment, and to then use this polymer in a MISPE protocol. The samples of interest were urine and effluent water from wastewater treatment plants (WWTPs). These particular samples were selected after taking into account both the interest for CBZ determination as well as the desire to demonstrate that the MIP is able to extract CBZ selectively from either complex or highly diluted matrices.

In addition to many other pharmaceuticals [18,19], CBZ has been found in effluent water; this is because CBZ is not eliminated efficiently from wastewater in WWTPs [20–22]. No other treatments are available to completely remove this compound from water either, so CBZ can easily reach the surface water where it is discharged to other media when this water is reused. CBZ is thus becoming a significant hazard for the general population and to the environment.

2. Experimental

2.1. Reagents and standards

For the syntheses of the polymers, methacrylic acid (MAA) was selected as functional monomer, divinylbenzene 80 (DVB- 80) as cross-linker and CBZ as the template molecule; all were obtained from Aldrich (Steinheim, Germany). 2,2'-Azobisisobutyronitrile (AIBN), from Acros Organics (Geel, Belgium), was used as the free radical initiator. It was anticipated that MAA, as a hydrogen bond donor and hydrogen bond acceptor, would interact with the urea group in CBZ. In addition, the potential exists for π–π interactions between DVB-80 residues and CBZ. All components involved in the polymerisation process with the exception of CBZ were purified before their use. To that end, MAA was passed through an alumina column and then distilled under reduced pressure, DVB-80 was passed through an alumina column and AIBN was recrystallised from methanol (MeOH). CBZ was used directly as obtained due to its high purity (98%).

The solvents used in liquid chromatography (LC) or MISPE were acetonitrile (ACN), dichloromethane (DCM), MeOH and acetic acid (HOAc), all from SDS (Peypin, France). DCM was also used as the porogenic solvent in the polymer syntheses. Ethyl acetate, toluene, acetone and phosphoric acid were from Merck (Darmstadt, Germany), tetrahydrofuran (THF) was from Probus (Badalona, Spain) and chloroform ($CHCl_3$) and sodium hydroxide (NaOH) were from Prolabo (Fontenai S/Bois, France).Water was obtained from a Milli-Q purification system (Molsheim, France).

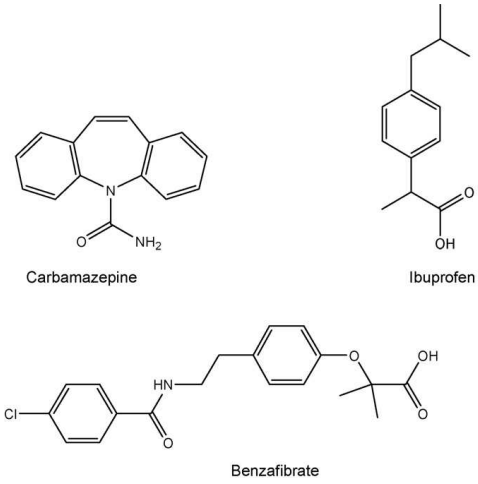

Fig. 1. Chemical structures of the compounds studied.

To assess the selectivity of the MIP, stock solutions of CBZ, benzafibrate (BZF) and (R/S)-ibuprofen (IBP) were prepared in MeOH at a concentration of 100 mg L^{-1}. Diluted solutions were prepared daily from these stock solutions. BZF and IBP were supplied by Sigma (St. Louis, USA). The chemical structures of CBZ, BZF and IBF are presented in Fig. 1.

2.2. Synthesis of molecularly imprinted polymer

CBZ (0.4 g, 1.69 mmol), MAA (0.584 g, 6.78 mmol), DVB-80 (4.415 g, 33.91 mmol) and AIBN (0.122 g, 0.7 mmol) were dissolved in dry dichloromethane (6.67 mL) in a 25 mL thickwalled, glass Kimax culture tube. Before polymerization, the solution was left on an ice-bath, sparged with oxygen-free nitrogen for 5 min (to remove oxygen) and sealed under a nitrogen environment. The tube was then left in a water bath set at 45 ºC for 24 h. The hard polymer monolith obtained was crushed, ground mechanically and wet-sieved using acetone. Polymer particles with sizes between 25 and 38 μm were collected. A non-imprinted, control polymer (NIP) was prepared in an analogous fashion to the MIP, albeit in the absence of CBZ. The isolated yields of the NIP and the MIP polymer particles with dimensions suitable for the subsequent LC and SPE work (25–38 μm) were 25% and 8%, respectively.

2.3. Equipment

For the chromatographic evaluation of the polymers, stainless steel LC columns (15 cm × 0.46 cm i.d.), one for each polymer, were slurry-packed with ~1.5 g of the 25–38 μm particles from each polymer using an air-driven, fluid pump (Haskel, Burbank, CA., USA). The solvent used for slurring and packing of the polymers was acetone. The chromatographic evaluation needed for the pre-screening work was performed using an SP 8800

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ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics, Mountain-View, CA., USA).

MISPE evaluation was performed in an off-line mode using 200 mg of each polymer (MIP and NIP) packed into 6 mL polyethylene cartridges (Symta, Madrid, Spain). The cartridges were connected to an SPE manifold (Teknokroma, Barcelona, Spain), which was connected, in turn, to a vacuum pump. The chromatographic instrument used was an LC-10AD binary liquid chromatograph equipped with a DGU-14A degasser, an injection loop of 20 μL, a CTO-10A oven and an SPD-10A UV detector. The LC instruments were from Shimadzu Corporation (Tokyo, Japan). The LC column used was a Kromasil 5 μm, 100 Å C_{18}, 250 mm × 4.6 mm from Teknokroma.

The liquid chromatograph–mass spectrometry instrument was an HP1100 series LC-mass selective detector (Agilent Technologies, Barcelona, Spain) with electrospray ionization. It was equipped with an automatic injector, a degasser, a quaternary pump and a column oven.

Effluent water from WWTPs and urine were filtered through 0.45 μm nylon membrane filters (47 mm diameter; Teknokroma) prior to injection on the LC.

The MIP was compared to a commercially-available polymer, Strata™ SDB-L Styrene-Divinylbenzene Polymer from Phenomenex (Torrance, CA, USA). The mass of sorbent used was 200 mg.

2.4. Chromatographic conditions

Prior to the chromatographic evaluation, the columns were conditioned overnight using a mobile phase comprising DCM:HOAc (95:5, v/v) in order to remove template and residual monomer from the polymers. For the chromatographic evaluation, 10 μL of a 10 mM CBZ solution and 2 μL of acetone (void marker) were injected, in turn, onto the MIP and NIP columns. Elution was carried out in isocratic mode, using chloroform as a mobile phase at a flow rate of 0.8 mL min^{-1}. The UV detector wavelength was set to 225 nm.

For the MISPE experiments, conditions for separating CBZ, BZF and IBF were established using a gradient of ACN and acidified water with H_3PO_4 to pH 3. The solvent gradient profile started with 40% ACN and reached 100% ACN within 3.5 min. From 3.5 to 6 min 100% of ACN was maintained, and from 6 to 8 min the ACN concentration decreased again until 40%. The flow rate was 1 mL min^{-1} and the UV detector was set to 225 nm. The column oven temperature was set to 30 ºC. The total running time for each analysis was 8.2 min.

Under these conditions, retention time for CBZ, BZF and IBP was 3.9, 4.5 and 5.6 min, respectively.

For LC–MS analysis, the conditions used were the same as those reported in a previous paper [20]. A binary mobile phase using water containing 0.5% of acetic acid (pH 2.8) as solvent A and ACN as solvent B was used to perform that analysis. The gradient was 18% B, which increased to 20% in 4 min, to 55% in 5 min, to 60% in 6 min, to 100% in 5 min, constant for 3 min, and finally decreased to 18% in 2 min. The temperature was kept at 30 ◦C, the mobile phase flow rate was 1 mL min^{-1} and the injection volume was 50 μL.

The conditions for MS were: positive ionization mode, nebuliser pressure at 40 psi, drying gas flow at 13 L min^{-1}, drying gas temperature at 300 ºC and capillary voltage at 3000 V. The fragmentation voltage used was 125 V. The chromatogram was acquired in selective ion monitoring (SIM) mode acquiring ions of m/z^+ 237 and 138.

2.5. MISPE conditions

Prior to any MISPE experiments, the polymers in the MISPE cartridges were washed several times with ACN, $CHCl_3$ and MeOH to remove template present in the polymer; the success of this extraction procedure was confirmed by injecting 20 μL aliquots of the MeOH eluent onto the LC. Thereafter, conditioning of the cartridges was required. This consisted of percolation of 10 mL MeOH through the cartridge and activation of the cartridge with 5 mL of water (adjusted to pH 3 with H_3PO_4).

For the loading step, the solutions used for evaluation of the MIP were 10 mL of water spiked with different concentrations (adjusted to pH 11 with NaOH). The pH of both types of sample (effluent water and urine) was also adjusted to pH 11 with NaOH and they were then filtered in order to remove all the precipitated material. The sample volume used in urine analyses was 2 mL and in the effluent water analyses was 100 mL.

For urine and effluent water samples, the washing step involved the percolation of 5 mL of water and 2 mL of ACN through the cartridge. As eluting solvent, 5 mL of MeOH was used.

3. Results and discussion

There is a growing requirement to detect yet lower concentrations of pharmaceuticals in an increasingly wide range of complex sample matrices, using, where possible, simpler analytical methods. This was our motivation for developing a new selective sorbent for CBZ.

3.1. Evaluation of the MIP

The MIP was evaluated chromatographically prior to any MISPE experiments in order to confirm the imprinting effect. Chromatographic evaluation was carried out by injecting 10 μL of 10 mM CBZ in $CHCl_3$ onto the MIP stationary phase. From these experiments, and analogous experiments involving the NIP, it was possible to calculate the retention factors (k': k'_{MIP} = 4.029 and k'_{NIP} = 0.845) and the imprinting factor (IF: IF = k'_{MIP}/k'_{NIP} = 4.765). This data shows that CBZ is bound more strongly to the MIP than the NIP under identical chromatographic conditions which, when taken together with an IF value of 4.765, is suggestive of a successful imprinting outcome. Independently of the LC experiments, however, the imprinting effect was also confirmed through MISPE experiments. Moreover, extensive peak-tailing of CBZ on the MIP was observed; this observation, which arises from the heterogeneous nature of the binding sites in a MIP, is wholly consistent with the elution profile expected for an analyte on an LC stationary phase imprinted using the same analyte.

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3.2. MISPE

To demonstrate that the MIP was capable of extracting CBZ selectively from any given sample matrix, two pharmaceutical compounds which may potentially be present in the same sample matrices as CBZ [20,23] were identified and selected for closer study. These compounds were benzafibrate (BZF) and ibuprofen (IBP). They were selected in preference to other pharmaceuticals because they contain polar functional groups that can potentially interact with the MAA residues present in the polymer. Similarly to CBZ, they are also aromatic.

Water was selected as the preferred loading solvent. This was our first choice of loading solvent because the samples of interest (effluent water and urine) were aqueous. From a practical perspective, when a cartridge can be loaded directly with the matrix of interest, better recoveries of the target analyte are obtained, enabling a lower detection limit to be achieved. When water spiked with all the compounds selected in this study was percolated through the polymer, complete retention on the cartridge of all of the analytes was achieved.

In order to favour the interactions between MIP and CBZ, a pH study was performed. The pH of the conditioning solvent and the pH of the sample were investigated across a pH range. Several solutions of water (10 mL) spiked with 2.5 mg L^{-1} of each compound at different pH values were prepared, some of which were adjusted to pH 11 (with NaOH) whereas others were adjusted to pH 3 (with H_3PO_4). Once the solutions were prepared, and prior to the loading step, percolation through the MIP of 5 mLof acidic (pH 3) or basic (pH 11) water was performed. After several trials, we concluded that the results were better when the MIP was activated at pH 3 and the sample percolated at pH 11 because, when other conditions were attempted, lower recoveries for all three compounds were observed. This may be because, under these particular loading conditions, MAA residues present in the polymer are non-ionised and able to establish polar interactions with CBZ.

MeOH and ACN were tested as elution solvents, with MeOH eventually being selected in preference to CAN because washing with 5 mL of MeOH permitted all compounds to be eluted from the cartridge. Some trials to improve the potency of the elution solvent were attempted, in order to decrease the overall volume of the elution solvent. This involved mixing MeOH in different ratios with acetic acid and applying the mixtures as washing solvents, but these experiments were not successful.

When water is used to load analytes onto an apolar polymeric cartridge, interactions between the analytes and the polymer are due mainly to non-selective, hydrophobic interactions. For this very reason, many compounds present in the sample are normally retained on the cartridge. To avoid interference of these compounds in the analysis of the target molecule, a clean-up step is normally included in the analytical protocol. To evaluate the usefulness of this step, and to demonstrate that the polymer synthesised was indeed imprinted, a comparative analysis between MIP and NIP was carried out. Two 10 mL solutions of each compound at 2.5 mg L^{-1} in water (pH 11) were percolated through the MIP and NIP.

Solvents tested to perform the cleanup step were $CHCl_3$, DCM, toluene, acetone, THF, ACN and mixtures of ACN:DCM. The best results were obtained when using 5 mL of ACN; when percolating 5 mL of ACN through the MIP, recoveries of CBZ were 60%, whereas the recoveries of BZF and IBF were <3%. When percolating the same volume (5 mL) of solvent through the NIP, the recovery of CBZ was <30% and the recovery of BZF and IBP was lower than 8%.

In order to emphasise further the imprinting effect demonstrated, an additional study comparing the MIP and a commercially-available polymeric cartridge (Strata™ SDB-L, Styrene-Divinylbenzene Polymer) was carried out. Since retention of compounds on this polymeric cartridge will be due primarily to non-selective, hydrophobic interactions instead of specific interactions, recoveries of compounds on this cartridge after the clean-up step should be very similar and very low for all compounds, in contrast to the MIP cartridge where high retention of CBZ and low recoveries for the rest of compounds were obtained. As expected, for the commercial cartridge the recoveries of all compounds were <10%. These results corroborate the earlier data referred to the imprinting effect.

In order to highlight the higher retention of CBZ on the MIP than on the NIP, 5 mL of ACN was used as the washing solvent but, for analytical reasons, instead of using 5 mL of ACN, conditions were sought which would lead to yet higher recoveries of CBZ. The modified conditions identified involved lowering the volume of the washing solvent (ACN) to 2 mL. In this case, the recovery of CBZ was now 85% and the recoveries of BZF and IBP were lower than 5% on the MIP.

Under the optimal conditions, the highest volume of sample that could be percolated through the MIP, without any losses in recovery, was set at 100 mL of water since, for this volume, recovery of CBZ was 85%. At higher volumes of the loading solvent, for instance 250 mL, recoveries decreased to 55%.

3.3. Real samples

Once the optimised MISPE experimental conditions had been established, extraction of real samples was performed to demonstrate the feasibility of applying the polymer to the extraction of CBZ from real samples. Since CBZ can be found both in samples of urine from people treated with this drug and in effluent water from WWTPs, these were the samples selected to perform the study. CBZ is a pharmaceutical which is difficult to eliminate in WWTPs [24] and, thus, it appears in effluent water and may reach the river or sea where these plants discharge. Urine samples are representative of highly complex matrices while effluent water from a WWTP is representative of complex matrices where very low concentrations of CBZ are expected to be found.

3.3.1. Urine samples

Since urine is a very complex matrix, a decrease in the volume of sample applied in the loading step was required. Instead of extraction of 10 mL of sample, as was the case previously, only 2 mL of urine was percolated

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through the cartridge. This decrease in volume was necessary because there are many compounds present that can compete with CBZ for binding to the polymer in the loading step, thereby leading to low recoveries of the target molecule.

Despite decreasing the volume percolated, many other compounds present in urine bound to the polymer, compounds that were eluted subsequently as a broad band appearing at the beginning of the chromatogram. In order to avoid this problem, an additional step was included in the MISPE protocol; this involved percolation of 5 mL of water before the clean-up with 2 mL of ACN. Despite the introduction of a new step in the whole process, no decrease in recoveries was observed. Fig. 2 shows the effect on the LC chromatograms of including the clean-up step in the MISPE procedure; chromatogram 2a was obtained in the absence of a clean-up step, whereas chromatogram 2b was obtained after a clean-up step. For the MIP loaded with 2 mL of urine (from healthy volunteers) spiked with CBZ at 2.5 mg L^{-1}, washed with 5 mL of water and 2 mL ACN, and eluted with 5 mL MeOH, the recovery of CBZ was 65%, whereas very small peaks were detected for BZF and IBP (recovery < 3%).

For CBZ quantification, a method validation was required. In order to set the method, urine from healthy volunteers was required. The urine was spiked with different concentrations of CBZ in order to test the linearity, repeatability, reproducibility and limit of detection of the method. The linear range was established between 0.05 and 24 mg L^{-1} with a determination coefficient (r^2) of 0.998. Repeatability and reproducibility between days, expressed as relative standard deviation (%R.S.D.) and $n = 3$, for a concentration of 0.5 mg L^{-1} were 3.1% and 12.6%, respectively. The limit of detection was set at 25 μg L^{-1}.

3.3.2. Wastewater samples

Since the concentration of pharmaceuticals in wastewater is expected to be lower than the corresponding concentrations in urine, higher sample volumes have to be extracted. Thus, the volume of sample selected was 100 mL.

In order to demonstrate that MISPE was suitable as an extraction technique for wastewater, 100 mL of effluent water from a WWTP, spiked with CBZ at 1 μg L^{-1}, was percolated through the polymer and analyzed by liquid chromatography–(electrospray) mass spectrometry using an identical chromatographic protocol to that described in a previous paper for the determination of a group of pharmaceuticals in wastewater [20]. Subsequent

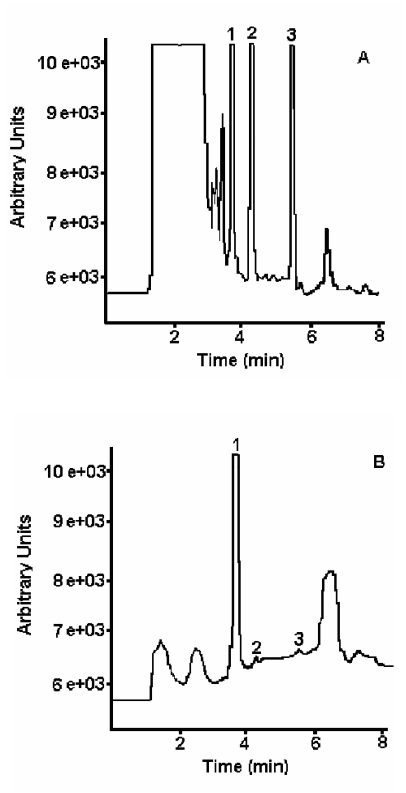

Fig. 2. Chromatograms obtained after percolation of 2mL urine spiked with 2.5 mg L^{-1} of each compound through the MIP, without (A) and with (B) a clean-up step comprising 5mL of water and 2mL of ACN. Peak assignment: 1: CBZ; 2: BZF, 3: IBP.

to the percolation of the sample and application of the clean-up step, 5 mL of MeOH was used in the elution step. The eluate was collected, evaporated to dryness under a N_2 stream and reconstituted in MeOH (500 μL). 50 μL of this solution was injected into the chromatographic system. The peak corresponding to CBZ was readily identifiable in the chromatogram and could be quantified easily (see Fig. 3, where the chromatogram obtained under selected ion monitoring acquisition is shown). Recoveries of CBZ present at 1 μg L^{-1} in this volume of sample were of 80%, and are similar to the values obtained in water. Therefore, the successful use of

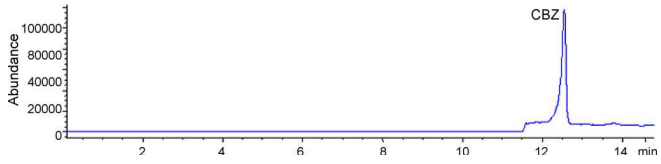

Fig. 3. Chromatogram obtained using LC–(ESI)–MS and SIM acquisition of ions $m/z^+ = 237$ and 138.

the MIP in the extraction of CBZ at low levels from effluent water has been demonstrated.

4. Conclusions

In this paper, a polymer imprinted for CBZ has been synthesised *via* a non-covalent molecular imprinting approach. The MIP has then been applied in a MISPE protocol, which enables the selective extraction of CBZ from samples, even when CBZ is present at low concentrations in chemically-complex samples. It has been demonstrated that the polymer is able to bind CBZ present in urine samples as well as samples where the concentration of CBZ is especially low, i.e., effluent water from a WWTP. CBZ recoveries of 65% were obtained when 2 mL of urine spiked with CBZ at 0.2 mg L^{-1} was percolated through the polymer. The limit of detection for urine samples was set at 25 μg L^{-1}. For samples from WWTPs (100 mL samples spiked at 1 μg L^{-1} and analysed by MS detection), recoveries of 80% were obtained, which is similar to the values obtained when the analogous extractions were performed directly from water.

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2.2.2 Synthesis by precipitation polymerisation of molecularly imprinted polymer microspheres for the selective extraction of carbamazepine and oxcarbazepine from human urine

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Synthesis by precipitation polymerisation of molecularly imprinted polymer microspheres for the selective extraction of carbamazepine and oxcarbazepine from human urine§

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ABSTRACT

Two molecularly imprinted polymers (MIPs), in the physical form of well-defined polymer microspheres, were synthesised *via* precipitation polymerisation (PP) using an antiepileptic drug, carbamazepine (CBZ), as template molecule, methacrylic acid as functionalmonomerand either divinylbenzene 80 (DVB-80) or a mixture of DVB-80 and ethylene glycol dimethacrylate (EGDMA) as crosslinking agents. The MIP obtained using DVB-80 alone as crosslinking agent (MIP A) had a narrow particle size distribution (9.5 ± 0.5 μm) and a well-developed permanent pore structure (specific surface area in the dry state = 758 $m^2 g^{-1}$), whereas when a mixture of DVB-80 and EGDMA (MIP B) were used as crosslinking agents, the polymer obtained had a broader particle size distribution (6.4 ± 1.8 μm) and a relatively low specific surface area (23 $m^2 g^{-1}$). The molecular recognition character of both polymers was evaluated by means of LC and then a molecularly imprinted solid-phase extraction (MISPE) protocol; CBZ was recognised by both polymers, and useful cross-selectivity for oxcarbazepine (OCBZ), which is the main metabolite of CBZ, also observed. In a detailed bioanalytical study, MIP A was selected in preference to MIP B since MIP A enabled a high volume of sample to be extracted such that lower limits of detection were achievable using this polymer. High recoveries of CBZ and OCBZ were obtained in a MISPE protocol when 50 mL of human urine spiked at 0.2 mg L^{-1} were percolated through MIP A (90% and 83%, respectively).

1. Introduction

Molecularly imprinted polymers (MIPs) are polymeric materials specially designed to offer valuable molecular recognition properties. This molecular recognition character has been very attractive in many different, enantiomeric separations and analytical applications, amongst others. Regarding analytical applications, MIPs have been applied successfully in many distinct ways; thus far, the main interest has focused on solid-phase extraction (SPE), more specifically the up-and-coming technique of molecularly imprinted solid-phase extraction (MISPE). In recent years, many different MIPs have been synthesised to selectively extract a diverse range of compounds from many different matrices [1,2]. When sorbents are applied in SPE protocols, for off-line SPE work tight control of the size and shape of the particles is not as critical as in liquid chromatography. Nevertheless, even in off-line SPE uniformity in size and shape of the particles is desirable, not only to enable reliable and reproducible packing of the cartridges, but also to improve the mass transfer between the mobile phase and the stationary phase, leading to an increase in the efficiency and effectiveness of the cartridge and therefore the analytical protocol.

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Many synthetic approaches towards imprinted polymer particles involve, in the first instance, the synthesis of an imprinted polymer monolith [3]. The monolith obtained then has to be ground and sieved to deliver a fraction of particles of the desired size for the intended application. Particles obtained in this manner are typically irregular in shape and size.

In order to overcome the limitations of the 'monolith approach' to imprinted particles, but also to increase the yields of useful particles, different methods aimed at the production of spherical imprinted particles have been explored. Some of these methods involve grafting procedures [4,5], whereas other methods involve emulsion [6,7], dispersion [8], suspension [9] and multistep swelling [10,11] polymerisation. However, in the majority of cases stabilizers and surfactants are required during the syntheses, additives which can contaminate the final products. Extensive and tedious optimisation of synthesis conditions may also be required.

Our preferred approach to the direct production of imprinted polymer particulates is normally precipitation polymerisation (PP) [12–14]. Compelling reasons for the routine use of PP include the fact that surfactant/stabilizer-free imprinted polymer microspheres can normally be produced in one single preparative step with excellent control over the particle size and particle size distribution [15,16]. This facile synthesismethod is being adoptedwidely, with the products being exploited in rebinding experiments [17–19] and affinity separations [20], for example. Such particles are clearly also well-suited for SPE work.

In this paper, we describe the use of precipitation polymerisation for the synthesis of two different MIPs in the form of imprinted microspheres with well-defined particle size and particle size distribution, and their subsequent use in MISPE protocols for the selective extraction of carbamazepine (CBZ) and its main metabolite, oxcarbazepine (OCBZ), from human urine. CBZ is a commonly used antiepileptic drug.

2. Experimental

2.1. Reagents and standards

Compounds used for the synthesis of the MIP and the nonimprinted polymer (NIP) were methacrylic acid (MAA) [functional monomer], ethylene glycol dimethacrylate (EGDMA) and divinylbenzene 80 (DVB-80) [crosslinkers] and CBZ [template molecule]. All these compounds were purchased from Aldrich (Steinheim, Germany). 2,2'-Azobisisobutyronitrile (AIBN), from Acros Organics (Geel, Belgium), was used as the free radical initiator.

All compounds used for synthesis were purified prior to use, and the solvents used were of analytical grade. To this end, MAA was passed through a column of neutral alumina and then distilled under reduced pressure; EGDMA was washed consecutively with 10% aqueous NaOH, water and brine, dried over $MgSO_4$, filtered and then distilled under reduced pressure; DVB-80 was passed through a column of neutral alumina and AIBN was recrystallised from methanol.

The solvents used for the chromatographic evaluations and the MISPE experiments were acetonitrile (ACN), acetone, methanol (MeOH) and dichloromethane (DCM), all from SDS (Peypin, France). Tetrahydrofuran (THF) was from Probus (Badalona, Spain), phosphoric acid (H_3PO_4) was from Merck (Darmstadt, Germany), and sodium hydroxide (NaOH) was from Prolabo (Fontenai S/Bois, France). Water was obtained from a Milli-Q purification system (Millipore, Molsheim, France).

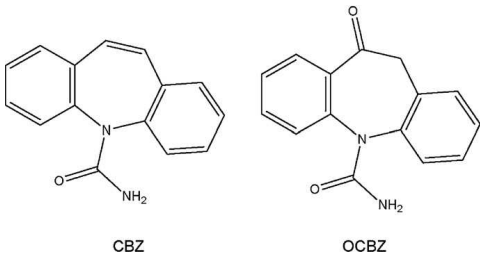

Fig. 1. Chemical structures of carbamazepine (CBZ) and oxcarbazepine (OCBZ).

Analytes used during the study were CBZ and its main metabolite, OCBZ (Sigma–Aldrich, St. Louis, MO, USA). A single stock solution (1000mg L^{-1}) of CBZ and OCBZ in water:MeOH (1/1, v/v) was prepared and kept in the fridge when not in use; diluted solutions were prepared from the stock solution daily. The chemical structures of CBZ and OCBZ are shown in Fig. 1.

2.2. Synthesis and characterisation of the polymers

The MIPs were synthesised as described in the following procedures. For MIP A: CBZ (0.302 g, 1.28 mmol), MAA (0.660 mL, 7.78 mmol), DVB-80 (3.640 mL, 20.44 mmol divinylbenzene, 5.03 mmol ethylvinylbenzene) and AIBN (0.185 g, 1.13 mmol) were dissolved in 100 mL of a mixture of ACN:toluene (75/25, v/v) in a 250 mL polypropylene bottle. The solution was sparged with N_2 for 5 min, sealed under an N_2 atmosphere and left to polymerise on a low-profile roller (Stovall, Greensboro, NC) housed inside a temperature-controllable incubator (Stuart Scientific, Surrey, UK). The temperature was ramped from room temperature to 60 ºC over a period of around 2 h and then held at this temperature for a further 24 h.

For MIP B: CBZ (0.198 g, 0.84 mmol), MAA (0.660 mL, 7.78 mmol), EGDMA (1.270 mL, 6.73 mmol), DVB-80 (2.190 mL, 12.30 mmol divinylbenzene, 3.03 mmol ethylvinylbenzene) and AIBN (0.156 g, 0.95 mmol) were dissolved in 133 mL of a mixture of ACN:toluene (90/10, v/v) in a 250 mL polypropylene bottle. The solution was sparged with N_2 for 5 min, sealed under an N_2 atmosphere and the monomers polymerised in the same fashion and under the same temperature regime as MIP A.

Two non-imprinted polymers, NIP A and NIP B, corresponding to MIP A and MIP B, were also synthesised using the same protocols used for the production of MIP A and MIP B, respectively, but without the addition of CBZ.

Once the polymerisations were complete, the particles were collected by filtration on nylon membrane filters and then Soxhlet extracted with MeOH for 24 h to remove CBZ and unreacted monomers. The particleswere then dried overnight *in vacuo* at 70 ºC and their size and specific surface areas determined.

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The specific surface areas of the polymers were measured by nitrogen sorption porosimetry performed on an ASAP 2020 Accelerated Surface Area and Porosimetry Analyzer (Micromeritics Instrument Corporation, Norcross, GA); specific surface areas were calculated using the BET method.

Scanning electron micrographs were obtained using a JEO JM-6400 Scanning Microscope (Peabody, MA). Particle size distributions were measured using Image J Launcher software [21].

2.3. Chromatographic instrumentation

To evaluate the affinity of the polymers for CBZ, four stainless steel LC columns (150 mm × 4.6 mm I. D.) were slurry packed with ~1.5 g of each polymer using an air-driven fluid pump (Alltech, Model 1666), with acetone as the slurrying and packing solvent, to give two imprinted columns (MIP A and MIP B) and two non-imprinted columns (NIP A and NIP B). Chromatographic evaluations were carried out using an SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA).

For MISPE analyses ~200mg of each polymer was packed, in turn, into empty 6 mL polyethylene cartridges (Symta, Madrid, Spain). The frits used in the cartridges were of 5 μm pore size (Applied Separations, Allentown, PA, USA) for MIP A, since the MIP A particles were >5 μm, and 2 μm pore size (Supelco, Bellefonte, PA, USA) for MIP B, NIP A and NIP B. The cartridges were connected to a vacuum pump through an SPE manifold (Teknokroma, Barcelona, Spain).

The liquid chromatograph used for the MISPE experiments was an Agilent 1100 series instrument equipped with an automatic solvent delivery system, a degasser unit, a binary pump, a column oven set at 30 ºC and a diode array detection (DAD) system (Agilent, Waldbronn, Germany). The LC column was a Kromasil 5 μm, 100 Å C_{18}, 250 mm × 4.6 mm from Teknokroma.

2.4. Chromatographic conditions

For the chromatographic evaluation of the imprinted and nonimprinted columns, 10 μL of a 1 mM solution of CBZ in ACN spiked with 2 μL of acetone were injected onto the columns. The mobile phase was ACN at a flow-rate of 1 mL min^{-1} and the UV detector wavelength was set at 225 nm. Imprinting factors (IFs) were calculated in the standard manner.

The LC conditions for the MISPE evaluations comprised a gradient of ACN and acidified water (pH~3) as mobile phase. This gradient started with 40% ACN and reached 100% ACN within 3.5 min. From 3.5 to 6 min 100% of ACN was maintained, and from 6 to 8 min the ACN concentration decreased again until 40%. The column temperature was kept at 30 ºC with a flow-rate of 1 mL min^{-1} and a detector wavelength of 225 nm.

2.5. MISPE conditions

The MISPE protocol involved activation of the cartridges with 10 mL of acidified water (pH~3) before loading with human urine (50 mL) under basic conditions (pH~12). For the clean-up step, 10 mL of water (pH~12) was used to remove the most polar compounds present in the sample. Methanol (5 mL) was used as the eluting solvent and quantification performed by direct injection of this methanolic fraction onto the LC column.

3. Results and discussion

Molecularly imprinted solid-phase extraction is a valuable sample pre-treatmentmethod exploited in both environmental analysis and bioanalysis work. In respect of such analytical applications, it is clearly desirable to have a MISPE protocol where there is a high recovery of analyte, but also a protocol where the extracts are as clean as possible. Furthermore, when aqueous samples are being handled it is advantageous if the samples can be applied directly to the sorbents, *i.e.*, if the sorbent is compatible with aqueous samples.

In respect of the desirable features of any synthetic protocol used to generate polymeric SPE sorbents, in view of the costs associated with multi-step synthetic strategies the more streamlined (efficient) and high yielding the synthetic route the better. Additionally, synthetic routes that can deliver beaded polymers particulates in one single step are particularly attractive, since this reduces chemical waste during sorbent synthesis and isolation, but also because beads pack efficiently into columns and cartridges. The ability to produce monodisperse beads in the low micrometre size range is becoming very attractive in view of the enhanced efficiency of separations arising from the use of such materials, as exemplified by the rise of UPLC-based technology.

3.1. Synthesis and evaluation of the MIP

The purpose of the present study was to develop a novel MISPE protocol for the extraction of CBZ, a commonly used antiepileptic drug, from human urine. As such, the sorbent used should ideally be aqueous compatible. The synthetic approach taken to deliver imprinted polymer beads was precipitation polymerisation, and as such we made use of the extensive in house experience which we have with this particular synthesis method. Two imprinted polymers (MIP A and MIP B) were prepared using CBZ as template and (an excess of) methacrylic acid as functional monomer, together with the corresponding non-imprinted polymers (NIP A and NIP B). Whereas MIP A and NIP A were prepared using DVB-80 as the crosslinking agent, MIP B and NIP B were prepared using DVB-80 and EGDMA in combination as crosslinking agents; the rationale here was to generate polymers which were somewhat less hydrophobic than MIP A and NIP A, and therefore more aqueous compatible.

Post-synthesis, the polymeric particulates were freed from template and unreacted monomers *via* Soxhlet extraction, the products dried to constant mass and the yields determined by gravimetric analysis. For MIP A and NIP A the isolated yields were 65% and 66%, respectively, whereas for MIP B and NIP B the isolated yields were 77% and 85%, respectively. Thus, good to excellent yields of potentially useful materials were generated in one single preparative step. Thereafter, the particles were imaged by scanning electron microscopy (SEM) and the specific surface areas of the particles measured by nitrogen sorption porosimetry.

From the SEM images (Fig. 2), we were pleased to observe that all four products had been produced and isolated in the form of discrete polymer beads with narrow particle size distributions and average diameters in the lowmicrometre size range as desired (MIP A, 9.5 ± 0.5 μm; NIP A, 4.1 ± 0.1 μm; MIP B, 6.4 ± 1.8 μm; NIP

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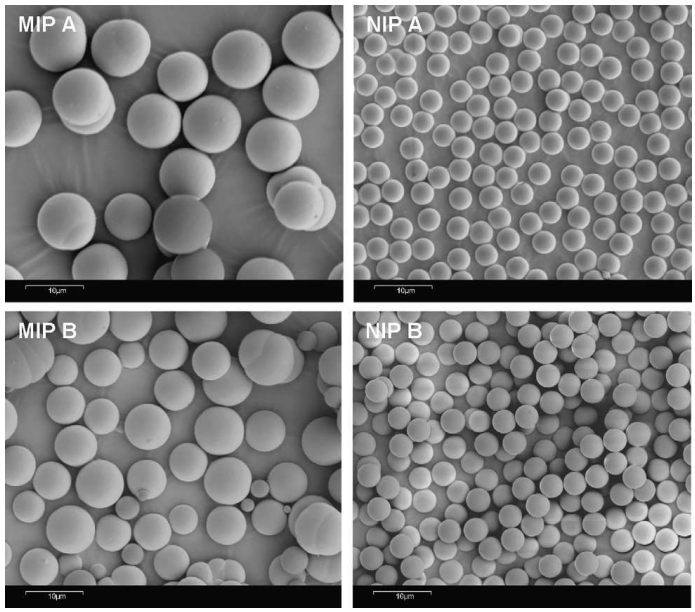

Fig. 2. SEM images of MIP A, NIP A, MIP B, and NIP B. Average particle diameters: MIP A, 9.5 ± 0.5 μm; NIP A, 4.1 ± 0.1 μm; MIP B, 6.4 ± 1.8 μm; NIP B, 6.1 ± 0.1 μm.

B, 6.1 ± 0.1 μm). The variations in average particle size reflect the facts that for a typical precipitation polymerisation the particle diameter of the final product is influenced by the nucleation stage of the reaction (the greater the number of particles nucleated, the smaller the average particle diameter for a given degree of monomer conversion) and the duration of the polymerisation (the average particle size increases with monomer conversion [of course, product yield is also related to monomer conversion]). Template can influence the nucleation stage of a precipitation polymerisation, analogously to the manner in which template can influence the nucleation stage of the production of an imprinted polymer monolith, thus it was not surprising that MIP A and MIP B did not have identical average particle diameters to their corresponding non-imprinted counterparts. In any case, having parity in particle size was not essential for the subsequent use of the particles in MISPE.

Nitrogen sorption porosimetry showed that MIP A and NIP A had well-developed pore structures in the dry state (MIP A, 758 ± 8m^2 g^{-1}; NIP A, 716 ± 8 m^2 g^{-1}). However, the inclusion of EGDMA in the monomer feed for MIB B and NIP B led to materials with low dry state specific surface areas (MIP B, 23 ± 0.8 m^2 g^{-1}; NIP B 3 ± 0.2 m^2 g^{-1}). Attempts were not made to prepare EGDMA-containing polymers with higher specific surface areas than MIP B and NIP B by, for example, changing the porogen, because it was found that the DVB-based imprinted polymer (MIP A) performed rather well when used as a MISPE sorbent for the direct capture of CBZ from fully aqueous samples (see later discussion).

In order to evaluate the feasibility of using the imprinted polymer microspheres as selective sorbents in MISPE, the imprinting factors (IF) for both MIP A and MIP B were calculated. From chromatographic evaluations of the polymers when packed into LC columns, the retention factors (*k'*) of CBZ on MIP A and NIP A were $k'_{MIP,A}$ = 27.1 and $k'_{NIP,A}$ = 8.2 respectively, which gave, in turn, an IF = $k'_{MIP,A}/k'_{NIP,A}$ = 3.3 for MIP A. From analogous experiments involving MIP B and NIP B, the retention factors of CBZ were $k'_{MIP,B}$ = 26.9 and $k'_{NIP,B}$ = 11.3, giving an IF = $k'_{MIP,B}/k'_{NIP,B}$ = 2.4 for MIP B, suggesting that MIP A had superior molecular recognition properties to MIP B. These IF values, when considered together with the asymmetric elution profile of CBZ on the imprinted columns, is already good evidence in support of successful molecular imprinting outcomes at the synthesis stage of the study. The polymerswere then evaluated in further detail through off-line MISPE experiments.

3.2. MISPE

In all MISPE experiments, water was the solvent of choice in the loading step since most environmental and biological samples are water-based matrices. In addition, in order to probe the selectivity, and possible cross-selectivity of the polymers, all the samples were spiked with CBZ and OCBZ.

In order to identify the optimal conditions for the retention of CBZ and OCBZ on both MIP A and MIP B, a pH study was performed. To this end, 10 mL of a standard solution spiked with 1 mg L^{-1} of CBZ and OCBZ in either acid media (pH~3) or basic media (pH~12) were percolated through both MIPs which had been activated previously with either acidic water (pH~3) or basic water (pH~12). This enabled up to four different trials for each polymer.

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For MIP A, recoveries of both CBZ and OCBZ were, in all cases, between 90 and 95%. High recoveries of OCBZ on a CBZ-imprinted cartridge were not unexpected because CBZ and OCBZ are very similar in chemical structure and 3D shape. For MIP B recoveries of CBZ were within the same range, but a decrease in recovery was observed for OCBZ when the sample was percolated in basic media. In this case, when the sample was percolated in basic media and the cartridge activated in acid media, recoveries of OCBZ were around 80%, whereas when the cartridge was activated in basic media the recoveries decreased to around 60%.

The high retention of CBZ and OCBZ on both MIP A and MIP B led us to believe that both polymers could potentially be used as selective extraction sorbents for the quantification of not only CBZ but also OCBZ present in aqueous-based samples. When applied to human urine samples, the sorbents may be potentially useful in monitoring the intake and metabolism of CBZ.

Various solvents were tested as eluting solvents: ACN, DCM, MeOH and acetone. From these four possibilities, MeOH was identified as the solvent of choice, since 5 mL of this solvent was sufficient to completely elute all the target analyte retained on both MIPs, whereas higher volumes of the other solvents were required to completely elute the same amount of analyte from the polymers.

For the MISPE clean-up step, both organic (DCM) and aqueous based (water at pH~12) solvents were evaluated.When using 5 mL of DCM as cleaning solvent, recoveries in the elution step after the MISPE protocol for CBZ were of 64% and 47% in MIP A and NIP A, respectively. The higher recovery of CBZ in MIP A compared to NIP A is further evidence of the higher affinity of MIP A towards the target molecule compared to the affinity of NIP A for the target.

However, the use of DCM, which is of course immiscible with water, required the cartridge to be dried under a stream of nitrogen. This necessitated an additional step to be set in place in the MIPSE protocol, thereby lengthening the overall analysis times, thus it was pleasing to observe that with water as the clean-up solvent, the MISPE extracts were acceptably clean and the analyte recoveries remained high. Thus, water at pH~12 was effective for this purpose so was used as the preferred clean-up solvent thereafter; an advantage is that a cartridge drying step was not required.

To evaluate the capacity of the MIPs in terms of the volume of sample which could be passed (percolated) through the cartridges whilst retaining high levels of analyte recovery, several solutions spiked with CBZ and OCBZ at 0.2, 0.1, 0.04 and 0.01 mg L^{-1} in 50, 100, 250 and 1000 mL of basified water, respectively, were percolated through the MIPs. For MIP A, high recoveries (>90%) of both compoundswere maintained up to 250 mL; for the 1000 mL sample volume the recoveries decreased to 70% for CBZ and 40% for OCBZ. For MIP B, recoveries were >90% for the low sample volumes (50 and 100 mL), but when 250 mL of water was percolated the recoveries fell to 67% for CBZ and 58% for OCBZ, most likely due to the low specific surface area of MIP B. Based on these results, MIP A was selected as the polymer of choice for the further development work with urine, and 250 mL of water selected as the sample volume to be percolated through the MIP. In passing, it is worthwhile noting that MIP A, which was prepared in beaded form by precipitation polymerisation, gave high recoveries of analyte at sample volumes considerably greater than the volumes which could be tolerated by cartridges packed with CBZ-imprinted polymer particles derived from crushed and ground polymer monoliths (>90% recovery using a 250 mL sample volume for the beads, *versus* 85% recovery of CBZ using a sample volume of 100 mL on the monolith-derived particles) [22].

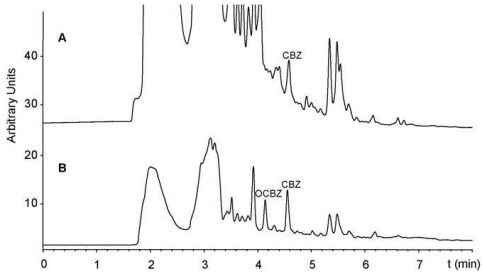

Fig. 3. Chromatograms of SPE extracts obtained after percolation of 50 mL of human urine spiked at 0.05 mg L^{-1} with CBZ and OCBZ through Oasis HLB (A) and MIP A (B) with a clean-up step involving 10 mL of water (pH~12).

3.3. Urine samples

Prior to the MISPE of urine samples, the pH of the samples was brought to basic conditions (pH~12) and the samples filtered through 0.22 μm pore diameter membranes to remove any precipitated material. Since MIP A bound CBZ and showed cross-selectivity for OCBZ, when this MIP was applied to human urine samples all the samples were spiked with CBZ and OCBZ.

Using the optimised MISPE protocol, the analyte recoveries from 50 mL of urine spiked at a level of 0.2 mg L^{-1} were 90% and 83% for CBZ and OCBZ, respectively. The recoveries were high under these conditions, thus a sample volume of 50 mL was used in the subsequent urine experiments because this volume is high enough to enable low limits of detection to be achieved.

The increase in both capacity and recoveries of analytes from water samples demonstrated in the present work compared to a previous report by our group [22], has also been observed for urine samples. Thus, the volume of human urine which can be percolated has been increased from 2 to 50 mL, and the recovery of CBZ has increased from 65% to 90%. This represents substantial and significant improvements to the MISPE protocol.

In MISPE applications, a cartridge clean-up step using an organic solvent is often required. However, when MIP A was applied to the MISPE of human urine, quantification of the analytes was achieved easily without interferences from the sample matrix, so a clean-up step using an organic solvent was omitted since no improvements in the chromatograms obtained was observed. Nevertheless, 10 mL of water (pH~12) was percolated through the cartridges prior to the elution step in order to suppress the broad band appearing at the beginning of the LC chromatograms; this broad band is ascribed to the highest polarity compounds in the sample. Inclusion of this clean-up step into the MISPE

protocol did not decrease the recoveries of either CBZ or OCBZ.

In order to validate the method for quantification of CBZ and OCBZ in human urine, linearity, repeatability, reproducibility and limit of detection (LOD) were calculated using 50 mL of human urine from healthy volunteers spiked with CBZ and OCBZ at different concentrations. The linear range was established between 1 and 0.02 mg L^{-1} for CBZ and between 1 and 0.04 mg L^{-1} for OCBZ, with a determination coefficient (r^2) of over 0.999 in both cases. The limit of quantification (LOQ) was 20 μg L^{-1} for CBZ and 40 μg L^{-1} for OCBZ.

To test the repeatability of the method for both compounds, the same volume of sample spiked with 0.05 mg L^{-1} of each compound gave values, expressed as relative standard deviation (%RSD) from three same-day replicates, of 1.3% for CBZ and 4.7% for OCBZ. The values for reproducibility from three different days, also expressed as %RSD using the same volume of sample and spiking concentration, were 3.5% and 7.1% for CBZ and OCBZ, respectively. The LOD for CBZ was set at 0.007 mg L^{-1}, whereas the LOD for OCBZ was 0.02 mg L^{-1} calculated as three times the signal to noise ratio. Comparing this data yet again with published data, [22] the capacities of the new polymers are higher, which means that the LOD can be lowered and the sensitivity of the analytical protocol improved.

In Fig. 3, the effectiveness of the MIP A in the selective retention of the compounds can be seen. Chromatogram A corresponds to the SPE protocol of 50 mL of human urine sample spiked at 0.05 mg L^{-1} with CBZ and OCBZ, using an Oasis HLB cartridge (Waters, Milford, MA, USA). Chromatogram B corresponds to the same volume and concentration for both CBZ and OCBZ using MIP A in the SPE protocol. In both cases a clean-up step using 10 mL of water (pH~12) has been included in the protocol. As can be seen, when the sample was percolated through the Oasis HLB cartridge, although CBZ could had been quantified, therewas no signal for OCBZ. Moreover, a clear improvement in the outcome of the chromatogram has been achieved when using the imprinted polymer as sorbent in the SPE protocol.

4. Conclusions

This paper describes the one-pot synthesis, in good to excellent yields, of porous molecularly imprinted polymers in beaded form which show selectivity of binding for a commonly used antiepileptic drug carbamazepine, and its main metabolite, oxcarbazepine. The beads are narrow in terms of their particle size distributions, they pack efficiently into columns and cartridges, and have average diameters in the low micrometer size range making them wellsuited to high efficiency separations. Rather significantly from the point of view of environmental analyses and bioanalyses, in use the polymers were found to be compatible with aqueous-based samples, including human urine.

A MISPE protocol has been developed for CBZ and OCBZ in human urine in which high recoveries of analytes (>80%) are achievable with urine sample volumes as high as 50 mL. The limit of detection of the analytical method was set at 7 μg L^{-1} for CBZ and 20 μg L^{-1} for OCBZ. Thus, the new method out-performed a published MISPE protocol for CBZ, both in terms of the LOD and the maximum sample volume which can be used routinely. This work sets in place the basis for a new bioanalytical protocol to detect the presence of an antiepileptic drug, and its main metabolite, in human urine.

Acknowledgements

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2.2.3 Discussion of Results

As can be seen in the papers presented, three different MIPs were synthesised under two different polymerisation protocols; two of them were obtained under the Precipitation Polymerisation protocol and the other one using Traditional Polymerisation. The aim of this study is to compare the performances of the two MIPs as SPE sorbents in MISPE protocols. Although two different MIPs with different monomer compositions were synthesised using the Precipitation Polymerisation protocol, to meet the goal of the study we selected the MIP with the same monomer composition as the MIP obtained under Traditional Polymerisation. Moreover, we were also pleased to observe that the one we selected to perform the comparison was the one with the highest surface area. Afterwards, the two MIPs selected were applied successfully to selectively extract the target compound (CBZ) from highly complex water-based matrices and to quantify it using liquid chromatography with a UV detection system. The results showed that higher recoveries for CBZ were obtained using both of the MIPs.

These results demonstrate that even though the rate of success for obtaining imprinted materials for any given molecule is higher with Traditional Polymerisation than with Precipitation Polymerisation, we could synthesise two different MIPs by two different polymerisation protocols and by using the same monomer composition. The reason for this is because many different template molecules and many different monomers can be used in Traditional Polymerisation to produce the final monolithic polymers desired. In contrast, when Precipitation Polymerisation is used to obtain the desired polymer, neither the composition of the polymers nor the synthetic conditions allow such variability.

The main difference between the two polymerisation protocols was the shape of the particles obtained. When the Precipitation Polymerisation approach was used, the final product consisted of spherical and smooth particles, all of which were within the narrow size distribution of the suitable range for MISPE applications. In the case of Traditional Polymerisation, the particles obtained were very irregular in shape and were bigger than the particles obtained by Precipitation Polymerisation. Fig. 14 shows a comparison of the particles derived from those two polymerisation protocols. Picture A shows the particles obtained from Precipitation Polymerisation and picture B shows the particles derived from Traditional Polymerisation.

When comparing both polymerisation protocols, an important advantage of Precipitation Polymerisation over Traditional Polymerisation is the reproducibility of the method. Even though a monolithic polymer of any given monomer composition can be produced several times, the final useful particles for MISPE applications derived from the Traditional Polymerisation protocol are, as stated previously, very irregular in shape and size (as can be seen in Fig. 14). In contrast to Traditional Polymerisation, once the best conditions for obtaining the polymer of interest are

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established using Precipitation Polymerisation, both the polymerisation yield and particle size distribution can be reproduced without major differences.

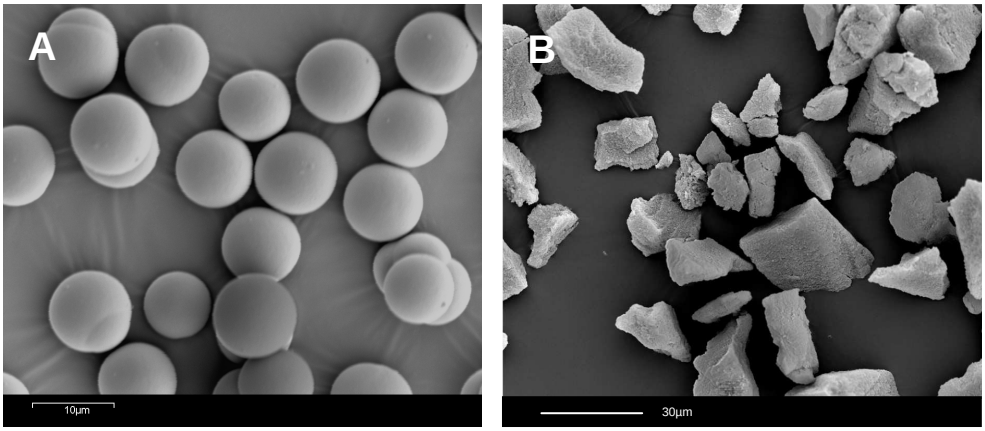

Fig. 14. Comparison of the different particle size and shape for MIPs obtained under Precipitation Polymerisation (A) and Traditional Polymerisation (B).

Another remarkable difference between these two polymerisation protocols is the time invested to produce a useful range of particle size for MISPE applications. In the case of Traditional Polymerisation, there is a long and tedious post-processing stage to achieve the useful particles once the final polymer is obtained. With Precipitation Polymerisation, the polymeric particles obtained are ready to be used immediately. Therefore, Precipitation Polymerisation is a much quicker and reproducible polymerisation technique than Traditional Polymerisation for obtaining imprinted particles of a suitable size for MISPE applications.

A consequence of the long post-processing of the polymer when using Traditional Polymerisation is that the yield of the useful range of particle size for MISPE applications is generally very low. In contrast, all of the product of the polymers obtained under Precipitation Polymerisation is generally within the required particle size in a moderate to good yield and this proved to be the case in the present study.

It is also worth noting that the presence of the template molecule during the polymerisation process in Precipitation Polymerisation protocols might have a huge effect on the outcome of the polymerisation product. This result, which was mentioned in the introduction, is due to the effect that the template molecule induces on the reactivity of the functional monomers used during the polymerisation process. In this case, two different MIPs were produced under the Precipitation Polymerisation protocol; one using DVB-80 exclusively as the cross-linking agent (MIP A) and another using a mixture of DVB-80 and EGDMA (MIP B). The reason for

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incorporating EGDMA was in order to decrease the hydrophobicity of the final product obtained.

A good way to see the different effects that the template molecule can produce during the synthesis of a polymer is to look at the results for the MIPs obtained under Precipitation Polymerisation. This can be done by comparing the dispersity of the particle size of the MIPs to their respective control polymers. In the case of MIP A, this dispersity was very similar to its control polymer (NIP A), whereas the dispersity of particles was higher for MIP B than for its control polymer (NIP B). However, the particle size for both MIP B and NIP B was very similar, whereas the particle size of MIP A (9 µm) was twice the size of NIP A (4 µm). At this stage, we were happy to observe such a big particle size for MIP A, because the normal reported particle size in literature ranges from 2 to 5 µm and, sometimes, even in the submicron range. This fact proves that particles over 5 µm can be obtained by this polymerisation protocol. This fact is highly desirable, especially for MISPE *off-line* applications, because it enables a proper packing of the cartridge with the mostly widely-used SPE fittings and prevents high backpressures in the system. It also enables a proper flow to pass through the particles and an effective mass-transfer of the analyte on to the solid support.

However, the effect of the template molecule on the final product obtained when comparing the synthesis of a MIP to its control polymer (NIP) is not always in this direction. From our own experience, the presence of a template molecule during the polymerisation process may lead to a reduction in the particle size, an increase in the dispersity of the final product obtained (as for the MIP B obtained by Precipitation Polymerisation), or gel formation.

Once the polymers were obtained, we assessed their ability to retain the target molecule by measuring the imprinting factor (IF). As derived from the value obtained for each of them, we concluded that all of the polymers synthesised were indeed imprinted. This demonstrates the success of the imprinting process.

Regarding the MIPs obtained by Precipitation Polymerisation, only MIP A was applied as a sorbent in the MISPE protocols because MIP B had a very low surface area and, therefore, the capacity of MIP B was expected to be considerably reduced compared to MIP A. Because of this, the comparison of the two polymerisation protocols was only carried out using MIP A.

When comparing both polymerisation protocols, another interesting result was the different breakthrough volumes obtained with the different MIPs although, as stated above, they had the same monomer composition.

To do that we used the same amount of sorbent for both of the MIPs obtained by Traditional Polymerisation and Precipitation Polymerisation and checked the maximum water volume that the sorbents could be percolated through. Under identical conditions, the MIP obtained under Traditional Polymerisation was able to

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completely retain the target molecule from 100 mL of water, whereas for the MIP obtained under Precipitation Polymerisation this volume was set at 250 mL. Moreover, when 1000 mL of water were percolated through the MIP obtained by Precipitation Polymerisation, the target molecule remained retained at over 70%. This high difference in capacity of the cartridges also supports the use of Precipitation Polymerisation over Traditional Polymerisation as the polymerisation protocol of choice for obtaining MIPs.

When assessing the polymers as selective sorbents for SPE protocols, we chose different control compounds for the MIP obtained from either Traditional Polymerisation or Precipitation Polymerisation.

For the MIP obtained by Traditional Polymerisation, we were interested in checking its ability for the selective retention of CBZ. We selected structurally related compounds to the template used, such as ibuprofen and benzafibrate, to check whether those compounds were also retained on the sorbent. From these results we realized that the active sites for CBZ formed within the polymeric matrix were well defined since only CBZ was retained on the sorbent.

To go one step further with the MIP obtained by Precipitation Polymerisation, the compound chosen for evaluating the selectivity of the polymer was oxcarbazepine (OCBZ), the main metabolite of CBZ which, in turn, is also used in the same way as CBZ and, therefore, can be present in the same samples and at similar concentration levels. In this case, since their structures are very similar (as can be seen on the paper describing the synthesis and use of this MIP), both CBZ and OCBZ were retained on the sorbent. However, CBZ was more strongly retained on the MIP than OCBZ, as can be seen from results obtained by percolating large water volumes. When 1000 mL of water were percolated through the sorbent, recoveries of CBZ were, as stated previously, recorded at 70% whereas for OCBZ the figure was 40%.

For real samples, as stated in the introduction to this chapter, we were interested in assessing the ability of the polymers to extract CBZ from human urine samples. In this case, an easy quantification of the analytes present in the sample was achieved by using liquid chromatography and UV detection systems together with both of the MISPE protocols developed. The most outstanding result was, as pointed out in the evaluation of the polymer, the higher capacity displayed by the MIP obtained under Precipitation Polymerisation. This polymer enabled the analysis of 50 mL of human urine while the MIP obtained under Traditional Polymerisation only enabled the analysis of 2 mL. Another interesting result concerned the recovery values obtained from this sample from the two MIPs. Recovery of the target analyte for the MIP obtained under Traditional Polymerisation was 65%, whereas the recovery for the MIP obtained under Precipitation Polymerisation was 90%. The higher capacity and higher recovery obtained by the MIP obtained under Precipitation Polymerisation further supports the advantages that this kind of polymerisation has to offer over Traditional Polymerisation.

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When we attempted to extract the target analyte from effluent water from wastewater treatment plants, we needed to use a more sensitive detectior, such as mass-spectrometer, because of the low concentration levels at which CBZ is present in this kind of matrix. Results from this experiment showed that the concentration of CBZ in the analysed samples was below the limit of detection.

From the data obtained in the studies above, we can conclude that Precipitation Polymerisation outperforms Traditional Polymerisation. Through the studies presented, we have been able to prove all the advantages claimed for Precipitation Polymerisation such as the reproducibility of the synthetic protocol, the high polymeric yield obtained and the readiness for using the particles obtained. However, the use of this technique is not always feasible because the presence of the template molecule during the polymerisation process has unpredictable effects and, sometimes, even prevents the intended polymeric product from being obtained. We also conclude that, despite its drawbacks, Traditional Polymerisation is a good protocol for synthesising MIPs and performing selective extractions.

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2.3 Use of a sacrificial spacer in the synthesis of the MIP

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When looking in literature, it is not difficult to realise that in the quest for obtaining better-imprinted materials, most attention has been focused on the polymerisation protocol, rather than on any other aspect of the imprinting process. Therefore, another area of interest in the present thesis was to exploit different approaches involved in the synthesis of MIPs.

Although different polymerisation techniques have been attempted as pointed out in the introduction part, less attention has been paid to improve the interaction between the template molecule and the functional monomer during the imprinting process and this is a key factor for obtaining more defined active sites within the polymer.

Up until now, the most widely-used interaction during the imprinting process between the template molecule and the functional monomer has been a non-covalent interaction, as described in the introduction. This is the simplest kind of interaction since it is based on equilibrium between a complexation form of these molecules and their free form in solution. For this reason, an excess of functional monomer is used in order to push the equilibrium to the complexation form. However, since complexation is a dynamic process, the excess of functional monomer added to the polymerisation mixture is not a guarantee that all the template molecules are equally complexed. This difference in the complexation process leads, in turn, to the formation of active sites with different affinities for the target molecule. Moreover, since polymerisation reaction is an exothermic process, the complex established between the target molecule and the functional monomer during the synthesis of the polymer can be deformed, thus leading to heterogeneity of the binding sites once the polymer is formed.

As mentioned in the introduction, Wulff *et al*. [1] introduced a different synthetic approach aimed at overcoming the deformation of the interaction of the template molecule and the functional monomer during the polymerisation process. This approach is known as the covalent approach but it has not been used in MISPE applications due to difficulties experienced during the synthesis and in the process of rebinding the target molecule on to the polymer.

An intermediate solution between the covalent and non-covalent approaches is the semicovalent approach. This approach tries to combine both the advantages of the covalent approach when synthesising the polymer and the advantages of the non-covalent approach when rebinding the target molecule. Therefore, highly defined binding sites left in the polymer with no excess of functional monomer are expected from the covalent bond between the functional monomer and the template molecule. Fast kinetic rebinding of the target molecule to the polymer and its fast release from the polymer is expected from the non-covalent retention of the target molecule to the polymer.

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Because of these considerations, we were particularly interested in checking the feasibility for obtaining an imprinted polymer using the semicovalent approach.

However, the main drawback in the semicovalent approach occurs during the process of rebinding the target molecule. The cavity generated in the polymer arises from the cleavage of a covalent bond and, because the target analyte is to be allocated within this cavity and to interact with the MIP through a hydrogen bond, since this kind of interaction requires longer distance than the covalent bond to be established, it is not difficult to envisage that the target molecule might not properly fit into the cavity. Therefore, low rebinding capacities are not unexpected.

In order to overcome this drawback, the solution proposed in bibliography is the introduction of a bonded labile functionality between the template molecule and the functional monomer. This is known as a sacrificial spacer. During the cleavage process of the template molecule from the polymer, this sacrificial spacer is also cleaved, which generates an extra space within the cavity for the proper allocation of the target molecule. This sacrificial spacer is normally a carbonate [2].

With this in mind, we decided to synthesise a MIP using the semicovalent approach, incorporate a sacrificial spacer and compare its performance to a MIP obtained by the non-covalent approach. Apart from that, we were also interested in exploiting the advantages that PP has over TP so we also attempted this polymerisation protocol in the synthesis of the polymer.

In the present study, the compounds we were interested in were esters of the *p*-hydroxybenzoic acid, more commonly known as parabens. These compounds are commonly used preservatives in many different products in close contact with the human population, such as pharmaceutical, food and body-care products.

Although recent information on their risk assessment concluded that their use is safe within the limits of current usage [3], some publications are warning of their possible effects as endocrine disruptors [4]. Therefore, these preservatives included in the so-called emerging organic contaminants, could present a serious hazard to human health and, due to their wide use and acceptance, their monitoring and surveillance is of prime interest.

Parabens are a large family of compounds that have the same core structure. Their differences lie in the different moieties attached to this core structure. For this reason and the fact that all parabens are potential endocrine disruptors, we aimed to synthesise a MIP with cross-selectivity for different parabens. The compounds used in this study were methyl 4-hydroxybenzoate (methyl paraben), ethyl 4-hydroxybenzoate (ethyl paraben), buthyl 4-hydroxybenzoate (buthyl paraben) and benzyl 4-hydroxybenzoate (benzyl paraben). We were interested in extracting these parabens from river water samples.

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Since the main interest of the study was to assess the advantages that the semicovalent approach offers over the non-covalent, we synthesised two MIPs under both of these approaches. Because we were interested in introducing a sacrificial spacer in the polymer during the semicovalent approach, the only commercially available compound with a carbonate structure and a suitable group for anchoring the functional monomer was 4-methoxycarbonylphenyl chloroformate, an analogue of methylparaben. This analogue, as shown in Fig. 15, has the same core structure as methylparaben and also incorporates the carbonate structure required to be a sacrificial spacer.

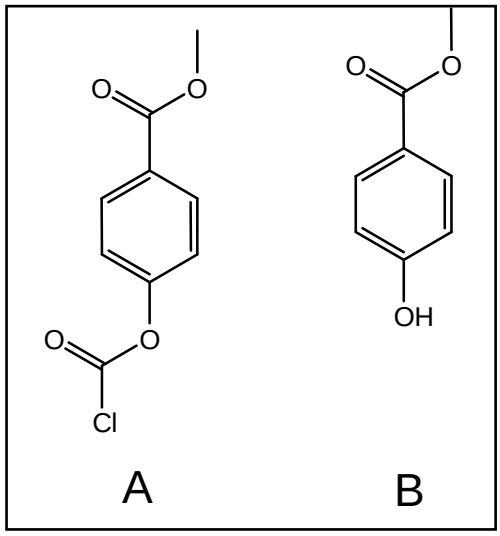

Fig. 15. Structures of 4-methoxycarbonylphenyl chloroformate (A); the analogue used for the target molecule methylparaben (B) in the synthesis of MIP 1.

For this approach, we synthesised the polymer under the PP protocol because of the good results previously obtained for the MIP targeted for carbamazepine.

The second MIP in this study was synthesised using buthylparaben and the TP protocol. We selected this template molecule because larger cavities could be generated within the polymer than with methylparaben. These larger cavities would improve the cross-selectivity of the polymer because they would allow different parabens to be retained on the MIP. This theoretical cross-selectivity for most of the paraben structures should happen because all parabens share the same core structure and interaction of the parabens with the MIP is through this core structure.

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The synthesis of this MIP was by TP since the synthesis by PP was not feasible. As pointed out in the introduction and in the previous chapter, the presence of a template molecule dramatically affects the outcome of the final polymerisation product. In this case, the presence of buthylparaben in a typical PP protocol leads to no obtention of the desired product.

The manuscript including the results derived from this comparison has been submitted for its publication to Analytica Chimica Acta.

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2.3.1 Synthetic Approaches to Paraben Molecularly Imprinted Polymers and their Applications to the Solid-Phase Extraction of River Water Samples

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Synthetic Approaches to Parabens Molecularly Imprinted Polymers and their Applications to the Solid-Phase Extraction of River Water Samples

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Abstract

In this paper we describe the synthesis, characterisation and use of two distinct molecularly imprinted polymers (MIPs) prepared using esters of *p*-hydroxybenzoic acid (parabens) as templates: one MIP was synthesised by precipitation polymerisation using a semi-covalent molecularly imprinting strategy with methyl paraben as the template/target (MIP 1); the second MIP was prepared in monolithic form through a conventional non-covalent molecular imprinting strategy, with butyl paraben as the template (MIP 2). MIP 1 recognized methyl paraben, showed cross-selectivity for other parabens analytes used in the study and higher affinity towards these compounds than did a non-imprinted control polymer. Similarly, MIP 2 demonstrated higher affinity towards parabens analytes than a non-imprinted control polymer.

For the analysis of environmental water samples, a solid-phase extraction (SPE) protocol was developed using MIP 2 as sorbent, and results compared to a SPE using a commercial sorbent (Oasis HLB). With MIP 2 as sorbent and butyl paraben as target, when percolating 500 mL of river water spiked at 1 μg L^{-1} through the SPE cartridge, and using 1 mL of isopropanol as cleaning solvent, a higher recovery of BuP and a cleaner chromatogram where achievable when using the MIP compared to the commercial sorbent.

Keywords: Molecularly imprinted polymer; Parabens; Precipitation polymerisation; Semicovalent approach; Sacrificial spacer

1. Introduction

Amongst the widest used antimicrobial preservatives in bodycare, pharmaceutical and food products are esters of *p*-hydroxybenzoic acid, referred to generally as parabens. Although parabens are used widely, surprisingly little attention has been paid to their potential hazards. The Final Report on Safety Assessment of Parabens concludes that the concentrations of paraben preservatives used in cosmetic products is safe [1], however some papers have appeared recently which describe the detection of parabens after dermal application [2] and warn about the possibility of parabens acting as endocrine disruptors [3]. Therefore, such compounds can be considered to be emerging pollutants and their determination is likely to become increasingly important. In this light, an analytical method involving any of the most commonly-used separation technique, such as LC, which allows their selective determination would facilitate any parabens studies, including toxicity studies. For solid-phase extraction (SPE) analytical protocol, amongst the best candidates as sorbents for performing selective extractions are molecularly imprinted polymers (MIPs). MIPs are synthetic sorbents designed to retain selectively the target molecule ideally regardless of the complexity of the matrix it is in [4, 5].

There are various synthetic protocols in the literature which describe the production of MIPs [6]. Although suspension polymerization [7, 8], swelling polymerization [9, 10], grafting procedures [11, 12] and precipitation polymerization [13, 14] are being used increasingly, many MIPs are still made using a simple polymerization strategy which delivers the MIP in the form of a monolith which can be broken down thereafter into particles as desired [15-17].

However, for some applications, having direct access to imprinted particles without the need for monolith grinding is highly attractive, for a variety of reasons, and several polymerization strategies can be used to achieve this.

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SYNTHESIS OF NOVEL MOLECULARLY IMPRINTED POLYMERS AND THEIR APPLICATION TO THE SOLID-PHASE EXTRACTION OF WATER-BASED MATRICES
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As an example, precipitation polymerization has been used to deliver spherical imprinted particles in a single preparative step, and is a particularly convenient method for MIP synthesis [13, 14, 18].

Regardless of the polymerization method used for their production, MIPs can be classified based on the nature of the interactions of the template with the functional monomer(s) during the polymerization process. The interactions can be either covalent or non-covalent, and the MIPs thus obtained are said to be produced using the covalent [19] or non-covalent [20] approach, respectively. However, there is third approach, known as the semi-covalent approach. This approach relies on a covalent bond(s) between the template and the functional monomer(s) during the polymerization, and a non-covalent interaction(s) between the target molecule and the MIP during the rebinding process [21]. In any case, whichever approach is taken determines the nature of the rebinding of the target analyte to the polymer.

Theoretically, MIPs obtained through either the covalent or semi-covalent approach offer certain advantages over MIPs obtained through the non-covalent approach [22]. Those advantages arise from the facts that no excess of functional monomer is used during the synthesis of the MIP (thereby diminishing non-specific interactions), there is likely to be more uniformity in the binding sites, and template bleeding is less likely to be an issue. By definition, rebinding of a target analyte to a MIP obtained by the covalent approach relies upon the formation of a covalent bond(s) between the polymer and the target whereas for a MIP obtained through the semi-covalent approach rebinding of the target analyte to the polymer relies upon the formation of (usually single) hydrogen bonds in the binding sites formed by the template molecule. This approach has its highest intrinsic value when care is exercised to ensure that the target is positioned precisely for optimal binding; a good way of achieving this aim is to introduce a sacrificial spacer [23, 24]. A sacrificial spacer is a labile functional group (often a carbonate) which links the functional monomer to the template molecule and which, once hydrolyzed, allows optimal positioning of the target molecule in the imprinted binding site.

It is within this context that we have synthesized parabens imprinted polymers using both conventional and precipitation polymerization methods, as well as semi-covalent and non-covalent imprinting approaches to investigate the influence of the synthesis conditions on the properties of the MIPs.

Thereafter, the MIP which displayed the best performance for the purpose of the analytical study was tested as an SPE sorbent to assess the feasibility of using such a material to extract paraben analytes from environmental water samples.

2. Experimental

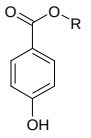
2.1. Reagents and standards

The compounds used to prepare the polymerisable template (PT) for the semi-covalent approach were 4-methoxycarbonylphenyl chloroformate (4-MCP) and 4-vinylphenol which was, in turn, obtained by hydrolysis of *p*-acetoxystyrene under basic conditions. For the synthesis of the corresponding non-imprinted polymer *p*-acetoxystyrene was used in place of PT. In this approach divinylbenzene-80 (DVB-80) was used as cross-linking agent for obtaining both polymers.

For the non-covalent approach, 4-vinylpyridine (4-VP) was used as functional monomer and ethylene glycol dimetacrylate (EGDMA) as cross-linking agent.

Once obtained the polymers, in order to evaluate their ability to retain selectively the parabens from real water samples, a mixture of four different parabens: methyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate (EtP), butyl 4-hydroxybenzoate (BuP) and benzyl 4-hydroxybenzoate (BzP) were used as test analytes. A stock solution containing all four parabens compounds at 1000 mg L^{-1} for each compound was prepared in water/methanol (1/1 v/v) and kept in the fridge; diluted solutions were prepared daily from this stock solution. Chemical structures of the parabens are presented in Table 1.

Table 1. Chemical structures of the parabens used in the study: methyl paraben, ethyl paraben, butyl paraben and benzyl paraben.

Core Structure	R	Name	Abbr.
	CH_3	Methyl paraben	MeP
	CH_2-CH_3	Ethyl paraben	EtP
	CH_2-CH_2-CH_2-CH_3	Butyl paraben	BuP
		Benzyl paraben	BzP

During the synthesis of all the polymers, the only compounds used as received were the parabens; all other compounds were purified prior to use. *p*-Acetoxystyrene was passed through a neutral alumina column to remove inhibitors. 4-VP was also passed through a neutral alumina column and distilled under reduced pressure. EGDMA was washed several times with 10% (w/v) aqueous NaOH, water and brine, dried over anhydrous $MgSO_4$, filtered and distilled under pressure. AIBN was recrystallised from methanol.

All the compounds involved in the semicovalent approach (*p*-acetoxystyrene, DVB-80 and 4-MCP), in the non-covalent approach (EGDMA, 4-VP) as well as the test analytes were supplied by Aldrich (Steinhem, Germany) and AIBN was from Acros Organics (Geel, Belgium). The solvents used during the study were 2-propanol, acetone, acetonitrile (ACN), hexane and methanol (MeOH) from SDS (Peypin, France). Chloroform, dichloromethane (DCM), phosphoric acid (H_3PO_4), tetrahydrofuran (THF) and toluene were from Merck (Darmstadt, Germany). Sodium hydroxide (NaOH) was from Prolabo (Fontenai S/Bois, France). Water was obtained from a Milli-Q purification system (Molsheim, France).

2.2. Synthesis and characterisation of the polymers

MIP 1 and NIP 1 were obtained by precipitation polymerisation using a semi-covalent approach whereas MIP 2 and NIP 2 were prepared in monolithic form by a

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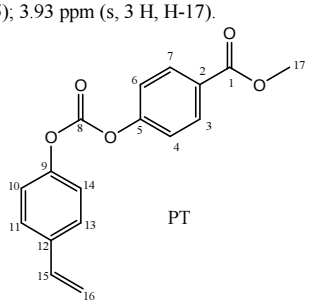
non-covalent approach.

For MIP 1, polymerisable template (PT) was prepared by treatment of 4-vinylphenol with 4-MCP in a fashion similar to that described by Whitcome *et al.* [23] during the synthesis of cholesterol imprints. 4-Vinylphenol (4-VPH) was obtained previously from hydrolysis of *p*-acetoxystyrene under basic conditions and was used without any additional purification. Fig. 1 shows a schematic representation of the synthesis of both PT and 4-VPH. For the preparation of PT, to 16 mmol (2.00 g) of 4-VPH in 60 mL of THF in a round-bottomed flask was added 16 mmol (3.60 g) of 4-MCP in 40 mL THF, dropwise and in the presence of triethylamine (Et_3N) (4 mL) and a few crystals of hydroquinone. Thereafter, the reaction was left overnight in an ice-water bath. Once the reaction was complete, the product was isolated by removing THF under reduced pressure and the crystals obtained further purified through a silica column.

The characterisation data for the polymerisable template were as follows; in the case of ^1H NMR (500 MHz, $CDCl_3$) δ_H = 8.13 ppm (m, 2 H, H-3, H-7); 7.47 ppm (m, 2 H, H-6, H-4); 7.38 ppm (m, 2 H, H-10, H-14); 7.27 ppm (m, 2 H, H-11, H-13); 6.71 ppm (m, 1 H, H-16 $_{Trans}$); 5.76 ppm (m, 1 H, H-16$_{Cis}$); 5.30 ppm (m, 1 H, H-15); 3.93 ppm (s, 3 H, H-17).



PT

For MS the mass calculated for the PT ($C_{17}H_{14}O_5$) was 298, which was found 298. The elemental analysis brought 67.91% C and 5.04%H. The melting point was within the range 98 - 101 ºC and the FT-IR data (KBr, cm^{-1}) 3519, 3420, 3080, 3046, 3006, 2958, 1765, 1724, 1630, 1604, 1508, 1443, 1435, 1412, 1275, 1216, 1107,890, 837, 778, 718.

For the production of MIP 1, PT (0.2150 g, 0.7 mmol) and DVB-80 (1.7850 g, 13.71 mmol) were dissolved in 100 mL of ACN:toluene (75:25) together with AIBN (0.0924 g). This solution was sparged with oxygen free nitrogen for five minutes on an ice-water bath, the container was tightly sealed and left on a low profile roller housed inside a temperature controllable incubator. Polymerisation thermally induced and the temperature ramped from room temperature to 52 ºC over two hours and held at this temperature for 46 h. For NIP 1, *p*-acetoxystyrene was used in place of PT. In this case, *p*-acetoxystyrene (0.1231 g, 0.7 mmol) and DVB-80 (1.8769 g, 14.41 mmol) were dissolved in 100 mL of ACN:toluene (75:25) together with AIBN (0.0972 g, 2 mol% w. r. t. polymerisable double bonds) and the polymerisation carried out under the same conditions as for MIP 1. Particles were imaged with a JEOL JM-6400 Scanning Microscope (Peabody, MA). Specific surface

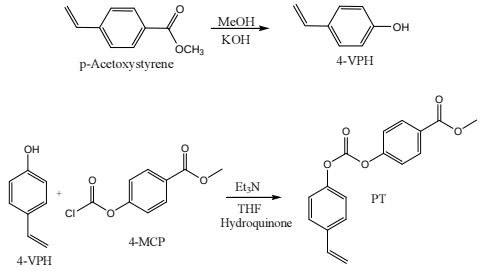


Fig. 1. Schematic representation of the synthesis of 4-vinylphenol (4-VPH) and the polymerisable template (PT).

areas for both MIP 1 and NIP 1 were determined after the hydrolysis treatment by BET treatment of sorption data generated by nitrogen sorption porosimetry analysis of the polymer using an ASAP 2020 Accelerated Surface Area and Porosimetry Analyzer (Micromeritics Instrument Corporation, Norcross, GA).

For MIP 2, BuP (0.221 g, 1.14 mmol) was used as template molecule, whereas no template molecule was present in the production of NIP 2. The other compounds used in the polymerisation process were used at the same molar ratios for both MIP 2 (1:4:20) and NIP 2 (0:4:20), and were: 4-VP (0.479 g, 4.56 mmol) as functional monomer, EGDMA (4.520 g, 22.8 mmol) as cross-linking agent and AIBN (0.164 g, 2 mol % w. r. t. polymerisable double bonds) as initiator. All of the components were dissolved in 6.67 mL of chloroform and left to polymerise in a Kimax culture tube, degassed for five minutes using oxygen free nitrogen, sealed under an N_2 atmosphere and left to polymerise in an oil bath. Polymerisation started at room temperature and reached 60 ºC in two hours and the polymer was held at this temperature for further 46 h.

Once the polymerisations were completed, the monoliths were ground in a mortar and wet-sieved using acetone and the fraction ranging from 25 to 38 µm was resuspended (x 3) in acetone to remove the fines and collected for Soxhlet extraction with MeOH to remove the template molecule and unreacted monomers.

All four polymers (MIP1, NIP 1, MIP 2, NIP 2) were obtained using conventional free radical polymerisation with 2,2'-azobisisobutyronitrile (AIBN) as initiator.

2.3. Chromatographic analysis

The MISPE extracts were analysed by LC using an LC-10AD binary liquid chromatograph with a DGU-14-A degasser unit, an STO-10 column oven and an SPD-10A UV detector measuring at 254 nm (from Shimadzu, Tokyo, Japan). The separation column used was a Kromasil 100 Å C_{18}, 250 x 4.6 mm from Teknokroma (Barcelona, Spain).

Chromatographic separation of the four parabens used in the study involved a gradient of ACN and acidified water (adjusted to pH ~ 3 with H_3PO_4) at a

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flow rate of 1 mL min^{-1} and a UV detector measuring at 254 nm. The gradient started with 50% of ACN and reached 70% ACN within 3 min. This composition was held until 6 min and raised up to 100% ACN within one min. From 7 min to 9 min the ACN concentration decreased again from 100% to 50%. The column was maintained at 30 ºC during all the analyses and the sample volume used was of 20 µL.

2.4 MISPE Conditions

For the MISPE study, 200 mg of each polymer was packed into 6 mL empty polyethylene cartridges (Symta, Madrid, Spain). For MIP 1 and NIP 1, the frits used were metallic frits of 2 µm pore size (Supelco, Bellfonte, PA, USA) whereas for MIP 2 and NIP 2 the frits were made of polyethylene had 10 µm pore size (Isolute, Mid Glamorgan, UK). The packed cartridges were connected to a vacuum pump through an SPE manifold (Teknokroma, Barcelona, Spain).

Two different MISPE protocols were developed using 200 mg of either MIP 1 or MIP 2 (and their respective control polymers) to assess the properties of the MIPs obtained by each polymerisation process.

For MIP 1, prior to the MISPE protocol, the cartridge was conditioned with 5 mL of MeOH and 10 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4). Afterwards, acidified water (adjusted to pH ~ 3 with H_3PO_4) was used as loading solvent, 1 mL of toluene as cleaning solvent, and the cartridge eluted using 5 mL of MeOH.

For MIP 2, the cartridge was conditioned with 5 mL of MeOH and 10 mL of basified water (adjusted to pH = 9.2 using 1 M NaOH) and the cartridge was loaded using basified water (adjusted to pH = 9.2 using 1 M NaOH), cleaned with 1 mL of 2-propanol, and eluted with 5 mL of MeOH. For real samples this methanolic fraction was evaporated to dryness under a stream of N_2 and reconstituted in 1 mL of MeOH prior to quantification.

A commercially-available sorbent, Oasis HLB (200 mg, 12 mL, N-vinylpyrrolidone-divinylbenzene copolymer) from Waters Corp. (Milford, MA, USA), was used for comparison.

River water samples were brought to pH = 9.2 and filtered through a 0.22 µm pore size nylon membrane prior to MISPE work.

3. Results and discussion

3.1 Synthesis of the MIPs

Two distinct strategies were adopted in the present study for the production of parabens imprinted polymers, in terms of the polymer format and the molecular imprinting approach used.

MIP 1 was synthesised by precipitation polymerisation using a semi-covalent molecular imprinting strategy with methyl paraben as the template target. The aim was to use precipitation polymerisation as a means of producing spherical imprinted particulates directly, and the semi-covalent approach to deliver binding sites with good overall uniformity. Our semi-covalent imprinting strategy exploited a sacrificial spacer [23] in the form of a carbonate linker (Fig. 3).

As stated previously, the PT was prepared by treatment of 4-vinylphenol with 4-MCP (see [23] for a cognate preparation of cholesterol-based polymerisable template). Thereafter, PT was copolymerised with DVB-80 (the cross-linker) under typical precipitation polymerisation conditions to deliver MIP 1 in the form of spherical polymer particulates. Cleavage of the template from MIP 1 was performed by refluxing the polymer overnight in a 1 M solution of NaOH in MeOH. Successful template removal was confirmed by FTIR spectroscopy of the polymer precursor and its hydrolysed derivative; bands appearing at 1782 and 1728 cm^{-1}, assigned to carbonyl stretching (carbonate and ester, respectively) were present in the spectrum of the precursor but not in the spectrum of its hydrolysed derivative. For the production of NIP 1, the non-imprinted control polymer, *p*-acetoxystyrene was used in place of PT. To this end, *p*-acetoxystyrene was copolymerised with DVB-80 under conditions similar to those used for the production of MIP 1, and the polymer obtained then hydrolysed using 1 M NaOH in MeOH. Cleavage of the acetoxy group was confirmed by FTIR spectroscopy (loss of the C=O str. band at 1769 cm^{-1}) following basic hydrolysis.

After polymerisation, the particles obtained were collected by filtration on a nylon membrane, washed several times with acetone and overnight dried in the vacuum oven leading to a polymerisation yield of 44% and 56% for the MIP 1 and the NIP 1, respectively. Afterwards, both the MIP 1 and NIP 1 particles were SEM imaged and we were pleased to observe that, as can be seen in Fig. 2, smooth spherical particles were obtained for both polymers. In the case of MIP 1, the particles showed narrower polydispersity than in NIP 1.

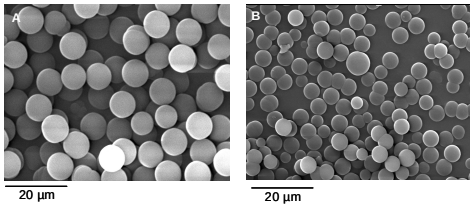

Fig. 2. Particles obtained in the production of (A) MIP 1 and (B) NIP 1.

The particle size were measured using Image J Launcher software [13] and the size for MIP 1 was of 8.1 ± 0.4 µm and the size for NIP 1 was of 5.0 ± 0.6 µm. Afterwards, both MIP 1 and NIP 1 were refluxed overnight in 1 M NaOH in MeOH to reveal the binding sites and, after the hydrolysis, the particles were imaged and sized again and, as expected, the sizes did not differ from those obtained before the hydrolysis. In this case, the size for MIP 1 was of 8.2 ± 0.4 µm and for NIP 1 was 5.0 ± 0.4 µm. The specific surface areas were 945 $m^2 g^{-1}$ and 590 $m^2 g^{-1}$ for MIP1 and NIP 1, respectively.

As can be seen in Fig 2, the size of the particles obtained for both MIP 1 and NIP 1 was within a narrow range of particle size and all of them displayed a smooth outer surface. However, the surface area of MIP 1 was far larger than the surface area for NIP 1.

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This is not an uncommon phenomena since the presence of a template molecule can dramatically affect the outcome of the polymeric reaction, as Sambe *et al.* reported on the synthesis of imprinted materials using S-nicotine as template molecule [25].

For the non-covalent approach, butyl 4-hydroxybenzoate (buthyl paraben, BuP) was used as template in the production of MIP 2. In this case, 4-vinylpyridine (4-VP) was used as functional monomer and ethylene glycol dimetacrylate (EGDMA) as cross-linking agent. The corresponding non-imprinted control polymer (NIP 2) was synthesised using the same functional monomer and cross-linking agent as for MIP 2, but in the absence of the template molecule. Once obtained, MIP 2 and NIP 2 were ground and wet-sieved with acetone and the fraction ranging from 25 to 38 µm collected and Soxhlet extracted with MeOH to remove template and unreacted monomers. The particles thus obtained were, as for MIP 1 and NIP 1, overnight dried in a vacuum oven and the yield of those particles was of 27% for MIP 2 and 17% for NIP 2.

3.2 Evaluation of the MIPs in solid-phase extraction

The molecular selectivity of all the MIPs and NIPs obtained in the present study were tested as sorbents in off-line MISPE protocols.

Since members of the paraben family share a common core structure, when evaluating the affinity of the MIPs for their respective templates we also introduced other members of the family (MeP, EtP, BuP and BzP) to probe cross-selectivity effects.

First of all, the optimal solvent to be used in the loading step was established. As can be seen in Table 2, 5 mL volumes of several solvents spiked at 2 mg L^{-1} with each parabens used in the study were percolated through the different MIPs, and the eluted fraction quantified for losses of the analytes in that step. From the data obtained, the best solvent with which to load the cartridge was found to be either acidified or basified water, since complete retention of all analytes was observed when using this solvent.

From the same data set, since when percolating the sample using MeOH as loading solvent no retention of the target analyte was observed, this solvent was eventually selected as eluting solvent. To set the volume of this solvent, several experiments using different volumes were performed and the optimal volume was found to be 5 mL since when volumes lower than 5 mL were used the elution of BuP was incomplete.

Once the optimal loading and elution conditions were established, we moved to optimise the clean-up step. A clean-up step is highly desirable when analysing real samples, since it allows compounds retained on a MIP through non-selective interactions to be removed. However, the clean-up step is also useful in highlighting the selectivity of a MIP for selected analytes.

As cleaning solvents hexane and dichloromethane (DCM) were applied to MIP 1 and NIP 1, but no clear differences between those two sorbents were observed when using either hexane or DCM. However, when using1 mL of toluene as the cleaning solvent, recoveries in the elution step from MIP 1, obtained after percolating

Table 2. Losses in the loading step when 5 mL of the following solvents, spiked at 2 mg L^{-1} with each paraben, were applied to the MISPE cartridge.

Solvent	MIP	MeP	EtP	BuP	BzP
Acidified Water	1, 2	0%	0%	0%	0%
Basified Water	1, 2	0%	0%	0%	0%
MeOH	1, 2	99%	99%	≥95%	≥97%
ACN	1	95%	92%	85%	86%
	2	80%	82%	75%	77%
DCM	1	70%	70%	98%	99%
	2	51%	62%	75%	77%

5 mL of acidified water (acidified to pH ~ 3 with H_3PO_4) spiked at 0.7 mg L^{-1} with each paraben, were 40%, 35%, 24% and 25% (n = 4, RSD <3.0 %) for MeP, EtP, BuP and BzP, respectively, whereas when percolating the same volume and concentration of sample through NIP 1 the recoveries after the elution step were 14%, 12%, 10 and 8% (n = 4, RSD < 11.0 % and in the same order as above), respectively. This data demonstrates not only the higher affinity of MIP 1 than NIP 1 for the parabens used in this study, but also brings to attention the enhanced affinity of MIP 1 for its template molecule than for the rest of the parabens used. This fact is directly derived from the highest retention of MeP on MIP 1 than the rest the compounds used which is due the synthetic approach taken. This synthetic approach enables a better allocation of this compound to the MIP cavity than the rest of parabens. As depicted in Fig 3, all the parabens are retained through the same point in their core structure but, since the cavity left in the polymer is only tailored for MeP, as the pendant moieties from this core structure increase, the steric impediments for those compounds do so. This fact explains the lower retention on MIP 1 for those compounds increasingly different to MeP.

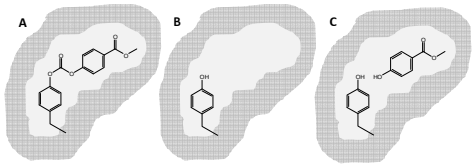

Fig. 3. Schematic representation of (A) MIP 1 obtained using the PT, (B) after cleavage of both the template molecule and the sacrificial spacer and (C) rebinding of the target analyte

Regarding MIP 2, a switch of the pH of the loading solvent from acid (pH ~ 3) to basic conditions (pH = 9.2 adjusted with 1M NaOH) was necessary in order to exploit the selectivity of the polymer. When loading MIP 2 under acid conditions there was no difference in the selective retention of the target analyte onto MIP 2 and NIP 2 whereas, under basic conditions, those differences arose when cleaning the sorbents with 1 mL of DCM (the same was true when the cleaning solvent was 2-propanol).

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More specifically, after percolating 5 mL of water (pH =9.2) spiked at 0.7 mg L^{-1} of each paraben and using 1 mL of DCM as cleaning solvent, recoveries obtained on MIP 2 for all the parabens under study were in excess of 97% for all analytes, whereas recoveries obtained on NIP 2 were 83%, 77%, 70% and 67% for MeP, EtP, BuP and BzP, respectively. Preferential analyte uptake by MIP 2 was observable when using 1 mL of 2-propanol in the washing step. When percolating 5 mL of basified water (pH=9.2) spiked at 0.7 mg L^{-1} with each parabens through MIP 2, recoveries for MeP, EtP, BuP and BzP were 32%, 26%, 67% and 25%, respectively, whereas when performing the same experiment but using NIP 2 instead, recoveries were slightly lower. The highest analyte retention on MIP2 was for BuP (BuP was also the template) therefore 2-propanol was used as the cleaning solvent for MIP 2 in subsequent experiments.

The different behaviour observed in retention of the analytes on MIP 2 and NIP 2 depending upon the pH of the sample can be explained by considering the cross-linking agent used in the synthesis of the polymers. Since EGDMA is the backbone in both polymers and this molecule has four O atoms, under acid conditions parabens can establish an effective H bond with the polymer and, therefore, there are no differences in retention on the MIP or the NIP. However, when the sample is percolated under basic conditions, parabens become ionic species and the highest retention on the MIP 2 than on the NIP 2 is due to the selective interactions between the parabens and the MIP.

The results obtained in MIP 2 and NIP 2 are in good agreement with those recently reported by Núñez *et al*. [26] where the authors synthesised two different MIPs for the selective extraction of parabens using either 4-VP of methacrylic acid (MAA) as functional monomer. In this case, for the MIP obtained using 4-VP as functional monomer, barely differences were observed between the MIP and the NIP, even after performing a clean-up step using toluene and mixtures of toluene and ACN, whereas for the MIP incorporating MAA as functional monomer, clear differences arose when performing a clean-up step using toluene.

When comparing the results reported herein with those in literature, selectivity of the MIP seems to be highly dependant on the functional monomer used during the synthesis of the polymer. When using 4-VP, interaction of the target analytes is not as effective as when using MAA. However, by fine tuning of experimental conditions, preferential uptake of the target analyte can be obtained even when using 4-VP, as has been demonstrated in the present study.

The markedly different results obtained when cleaning the cartridge with either 1 mL of DCM or 1 mL of 2-propanol brings about the possibility of exploiting either the cross-selectivity of MIP 2 for the parabens family (when using 1 mL of DCM) or the selectivity of MIP 2 for BuP (when using 1 mL of 2-propanol). Since we desired selectivity from MIP 2, the final conditions established for the MIP 2 MISPE protocol were percolation of the sample under basic conditions (pH = 9.2), clean-up using 1 mL of 2-propanol and elution using 5 mL of MeOH.

Once the optimal conditions for the MISPE protocols for both MIPs were established, we established the maximum volume of sample which could be percolated through the cartridges without analyte losses. This volume was set at 500 mL for MIP 1 and MIP 2, since recoveries from ultrapure water for all the analytes were greater than 98% for both MIPs when no clean-up step was performed. However, when a clean-up was performed in MIP 1 involving 1 mL of toluene, recoveries for MeP, EtP, BuP and BzP were of 39%, 30% 18% and 14%, respectively, whereas when a clean-up step was performed in MIP 2 with 1 mL of 2-propanol, the recoveries obtained were of 28%, 22%, 67% and 28%, respectively and in the same order as above. From those values, similar recoveries were obtained from either 500 mL or 5 mL of ultrapure water and, therefore, the maximum volume was set at 500 mL.

Overall, when comparing the two polymerisation protocols taken in this study we can confirm that, when feasible, Precipitation Polymerisation is a more suitable polymerisation technique to produce imprinted material of suitable size for MISPE protocols in a single preparative step and in a good polymerisation yield. It is also well-worth to note that the spherical materials produced enable a better packing of the cartridge than the irregular material derived from Traditional Polymerisation.

Regarding the synthetic approach taken, the semicovalent approach delivers a more defined active sites, as derived from the different recoveries for the parabens used in this study obtained in either MIP 1 or NIP 1

However, in some cases, a single interaction point might not be enough to achieve the recoveries yields expected in the MISPE protocol as derived from recoveries obtained in MIP 1. For the non-covalent approach the highest retention on MIP 2 can be attributed to the excess of functional monomer used in the production of the polymer. This excess can be a drawback since may lead to increase the number of non-selective interactions, as was the case of the comparison of MIP 2 and NIP 2.

3.3 Application to real samples

The sample we were interested in was river water since this detection of contaminants is of prime interest not only for the proper development of the environment but it is especially important when this water is going to be in close contact with human population. In this study, only MIP 2 was used due to the more effective clean-up step (with 1 mL of 2-propanol) achievable.

Due to the high capacity exhibited for MIP 2 during the evaluation of the polymers, our aim was to extract the analytes of interest from 500 mL of river water. To this end, river water was brought to pH = 9.2 and filtered through a 0.22 µm pore diameter nylon membrane. Afterwards, a MISPE protocol involving 500 mL of river water spiked at 60 µg L^{-1} was compared to the same MISPE protocol involving non-spiked sample. To decrease even further the limit of detection, the eluted fraction from the MISPE protocol was

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evaporated to dryness under a stream of N_2. Under these conditions, the MISPE protocol developed enabled the efficient extraction of up to 1 µg L^{-1} of BuP and BzP. However, at this concentration level, quantification of neither MeP nor EtP was possible since the corresponding chromatographic peaks were masked by the matrix components whereas for BuP and BzP, recoveries obtained from this volume and concentration level were of 58% and 27%, respectively (similar to their recoveries in ultrapure water).

The analysis of such volume of real sample is rather unusual for a MISPE protocol, since the normally used volumes range in the low millilitre range due to the generally low capacity exhibited for MIPs.

To assess the overall value in using MIP 2 to extract BuP from real samples, a comparison with a commercially-available hydrophilic-lypophilic balanced sorbent (Oasis HLB) was performed. For this comparison, 500 mL of river water samples spiked at 1 µg L^{-1} were percolated through both sorbents. In order to achieve optimal retention on the Oasis cartridge the sample was acidified (to pH = 3.0), whereas for MIP 2 the sample was basified (to pH = 9.2).

As can be seen in Fig. 4, after analyzing 500 mL of Ebre river water spiked at 1 µg L^{-1} with each parabens used in the study using either the MISPE protocol developed or a SPE protocol for the Oasis HLB sorbent, quantification of neither MeP nor EtP was achieved for either cartridge. In contrast, both BuP and BzP were retained on both cartridges. However, the use of the MIP as sorbent, enabled a cleaner extract to be obtained thus facilitating the quantification of the analytes.

Regarding the concentration normally found for parabens in bibliography, this concentration is lower than the concentration levels reported in the present paper. However, those concentration levels have been achieved using mass-spectrometry as detection system and this detection system has serious limitations when detecting parabens due to the high ion suppression suffered by those compounds. As example, our group recently reported a paper reporting detection and quantification of personal care products (including MeP, EtP and BuP) [27] and, although the LOD was found to be 1 ng L^{-1} in 500 mL in river water, there was a huge (>70%) ion suppression for MeP and also very high for EtP (40%) and BuP (15%). Another example of this effect on this kind of compounds was reported by González-Mariño *et al*. [28] when comparing two different mass-spectrometers for the detection of several household biocides, including parabens, and parabens turned out to be compounds with the highest ionic suppression. In the above mentioned cases, the sorbents used in the SPE protocol were Oasis HLB, as the commercial cartridge used for comparison in the present study.

As can be seen in Fig. 4, when comparing the MISPE protocol of river water samples developed in this paper to a conventional SPE using a commercially-available sorbent, the cleaner chromatogram obtained with the MISPE protocol would facilitate lowering the limit of detection for the parabens under study due to the decrease in the ion suppression derived from all the organic content retained on the commercial sorbent.

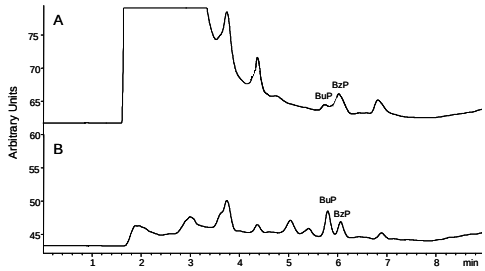

Fig. 4. Comparison of chromatograms obtained after extraction of 500 mL of Ebre river water spiked with 1 µg L^{-1} with each paraben under study in (A) conventional SPE using Oasis HLB as sorbent and (B) after the MISPE protocol developed using MIP 2.

4. Conclusions

Two different polymerisation methods, precipitation polymerisation to give beads and conventional polymerisation to give monolith, and two distinct molecular imprinting approaches, semi-covalent and non-covalent approaches, have been used successfully to produce parabens imprinted polymers.

Since recoveries of the analytes of interest were higher in MIP 2 than MIP 1, MIP 2 was further used to perform the extraction from real samples. Moreover, the high capacity exhibited by MIP 2 enabled the efficient extraction of 1 µg L^{-1} of BuP and BzP from 500 mL of river water samples and at similar recoveries to those obtained in ultrapure water.

When analysing 500 mL volume of river water samples spiked at 1 µg L^{-1} with several parabens, the molecularly imprinted sorbent enabled higher analyte recovery and a cleaner chromatogram to be obtained than for a commercial non-imprinted sorbent. Therefore, when a mass-spectrometry was used for detecting lower concentration levels of the analytes of interest, the use of the present MIP as sorbent for a SPE protocol, would considerably improve the quantification of the analytes due to the expected decrease in the typical ion suppression of parabens when they are in highly complex matrices.

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2.3.2 Discussion of Results

As can be seen in the previous paper, two different MIPs - using both different polymerisation methods and synthetic approaches - have been developed and their affinities to their target molecules assessed. Due to the high capacity exhibited for one of them, this polymer was applied for the selective extraction of parabens from river water samples.

The first MIP (MIP 1) was obtained under Precipitation Polymerisation using the semicovalent approach, whereas the second MIP (MIP 2) was obtained under Traditional Polymerisation using the non-covalent approach.

For MIP 1, we wanted to combine both of the advantages from precipitation polymerisation and the semicovalent approach. Therefore, uniformity among the particles obtained, a better packing of the cartridge and improved mass transfer of the analyte were expected from precipitation polymerisation, whereas uniformity in the cavities and binding sites was expected from the semicovalent approach adopted. In this case, the analogue of the template molecule required was the commercially-available 4-methoxycarbonylphenyl chloroformate.

In MIP 2, Traditional Polymerisation was the polymerisation protocol of choice because the synthesis of the polymer by Precipitation Polymerisation was not feasible. This is evidence that the presence of a template molecule might lead to the product not being obtained. In this case, buthyl 4-hydroxybenzoate (buthyl paraben) was selected as the template molecule.

When comparing the synthetic product obtained for MIP 1 and MIP 2, the results agreed with those reported in chapter 2.2 when synthesising a MIP using CBZ as the template molecule by either Precipitation Polymerisation or Traditional Polymerisation. In this case, again, the dispersity of the particle size for the MIP obtained by Precipitation Polymerisation was narrower than for the MIP obtained by Traditional Polymerisation and the polymerisation yield obtained for the MIP obtained under Precipitation Polymerisation was also higher than that obtained under Traditional Polymerisation.

However, in this case, for the polymers obtained under Precipitation Polymerisation, the dispersity of the particle size displayed by MIP 1 was higher than for its control polymer (NIP 1) and the particle size was lower for MIP 1 than for NIP 1. This is illustrative of the different and unpredictable effect that the template molecule can have on the polymerisation process.

Once the polymers were obtained, their ability to retain the target analytes was assessed by packing empty SPE cartridges with either MIP 1 or MIP 2.

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In this case, as pointed out in the introduction to this chapter, we were interested in demonstrating that the MIP could extract not only the template molecule used but also some other related structures. To this end, we included methyl, ethyl, buthyl and benzyl paraben as the target molecules for the MISPE protocol since all of them are used for the same purposes and are present in the same kind of matrices.

When developing the MISPE protocol, we were pleased to observe that water was a suitable solvent to load the cartridge with because, in some cases, the high polarity of this solvent prevents the retention of the target compounds on the cartridge. In our case, this was a good result because water was the sample we were interested in for extracting these compounds.

In order to reveal the selectivity of MIP 1, several solvents were used in the clean-up step once the cartridge had been loaded with water. As seen in the paper, from all the solvents used in this step, only toluene revealed the selectivity of the polymer.

From results obtained after using toluene as the cleaning solvent, recoveries of the target analyte were rather low. However, a clear difference was observed between MIP 1 and NIP 1. This difference, regarding the retention of the analytes, clearly points to a better recognition of the compounds of interest on MIP 1 rather than on NIP 1, thus demonstrating the good imprint effect left on MIP 1. The low recovery obtained on this MIP can be explained by the approach taken when this sorbent was synthesised. Because of this approach, a single active point within the cavity of the MIP is obtained and the analyte must be perfectly aligned to this active site to establish a proper retention.

An interesting result from this experiment is the steric impediment effect observed with this MIP. This effect was clearly observed when using different parabens. Since the size and shape of the cavity left in MIP 1 was designed for methylparaben, when larger molecules were to be allocated within this cavity, they could not fit properly. This conclusion was evidenced by the poorer retention on this MIP as the pendant moieties in these structures were increasingly dissimilar to the methyl group of methyl-pareben.

The MIP 2 sorbent was obtained by Traditional Polymerisation instead of Precipitation Polymerisation since the monomer composition selected for synthesising the polymer did not allow discrete particles to be obtained by Precipitation Polymerisation. Therefore, Traditional Polymerisation was selected as the polymerisation protocol and a monolithic polymer was obtained. This also proves that conditions for Precipitation Polymerisation are very specific and that Traditional Polymerisation is more robust when imprinting any given molecule.

In order to see the differences between the MIP 2 and NIP 2, a switch in the pH of the sample in the loading step was required. This switching was necessary due to the cross-linking agent used in the synthesis. Since the cross-linking used was ethylene glycol dimetacrylate (EGDMA), many non-selective interactions could be

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established between both the MIP 2 and NIP 2 and the target analytes when the sample was percolated under acid conditions, thus masking the imprinting effect left on MIP 2.

When the cartridge was loaded under basic conditions, two different behaviours were observed, which depended on the solvent used to perform the clean-up step. When the solvent used was dichloromethane (DCM), all the analytes were preferentially retained on the MIP rather than on the NIP. When 2-propanol was used, the most widely retained analyte was buthylparaben which was, in turn, the template molecule used during the synthesis of MIP 2.

The results obtained with MIP 2 are in accordance with those recently reported by Núñez *et al.* [5]. In that case, the authors synthesised several MIPs targeting paraben compounds and used either 4-vinylpyridine (4-VPy) or methacrylic acid (MAA) as functional monomers to obtain the MIPs. The authors reported very good results and important differences were observed between the MIP and the NIP when the functional monomer used for their synthesis was MAA. When the functional monomer used was 4-VPy, their results were similar to those reported in our study. The big difference observed between these results is directly related with the selection of the functional monomer during the synthesis of the MIP.

Even though there is a difference between MIP 2 and NIP 2 as reported in the present study, we assessed the performance for extracting paraben compounds of the MIP obtained against a conventional sorbent. As shown in the paper, the MIP outperformed the conventional sorbent in terms of selectivity when using LC-UV. Furthermore, it is expected that, if the extracts obtained from MIP 2 were to be analysed using mass spectrometry, the cleaner chromatograms obtained when using MIP 2 – instead of the conventional sorbent - would enormously facilitate the quantification of parabens since an important decrease in the typical ion suppression of this detection method would be expected. It is worth noting that parabens are particularly difficult to quantify in highly complex matrices (such as river water) due to the fact that the compounds suffer high ion suppression [6,7].

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2.4 Improvement of using multiple hydrogen bonding functional monomers

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In this study we were interested in synthesising a MIP using a specially designed functional monomer to match the functionalities of the template molecule in order to obtain the best possible interaction between the target molecule and the MIP obtained and, therefore, delivering a MIP with improved selectivity towards the target molecule. The synthetic approach used was Precipitation Polymerisation due to the inherent advantages this polymerisation method has over other existing ones, as mentioned in previous chapters.

Although the strategy of tailoring the functional monomers to the functionalities of the target molecule and the advantages that this approach brings has been described in bibliography, this strategy is not widely used for synthesising MIPs. There are several reasons for this but the most important is that to synthesise a functional monomer requires highly specialised skills and a lot of time and effort, making the process of obtaining a MIP time consuming and difficult. Some other reasons involve the fact that, for most of the templates used, it is not always feasible to synthesise a tailor-made functional monomer. However, this approach has several important advantages over protocols that use commercially available functional monomers. These include a higher affinity of the polymer obtained towards the target analyte, a higher number of specific interactions established between the target molecule and the MIP, the use of a smaller excess of functional monomer, and a higher homogeneity in the active site formed within the MIP. The group of Sellergren has extensively made use of this approach with very good results in the extraction of different compounds, such as riboflavin [1] or different β-lactamic antibiotics [2] from complex matrices.

Under this perspective and since the template we were interested in has a specially suited structure for this strategy, we decided to make use of it to synthesise a MIP and assess the feasibility of this approach.

The compounds we were interested in were barbiturates, which are used as tranquiliser compounds. The structure of barbiturates, is highly symmetrical, with repetition and arrangement of its functionalities in a special fashion. This makes the structure very suitable for developing a complimentary functional monomer.

By looking closely at the core structure of barbiturates, it can be seen that this is a cyclic amide in which all its functionalities are in β-position, all of which are able to establish hydrogen bonding in an acceptor-donor-acceptor (ADA) fashion [3]. Therefore, the target functional monomer should also have its functionalities in β-position and in a complimentary fashion to the template molecule, *i.e*. donor-acceptor-donor (DAD). The functional monomer which best suits all of these requirements is 2,6-*bis*-acrylamidopyridine (BAP). To synthesise the molecule to be used as the functional monomer we adopted the protocol reported by Kano *et al*. [4].

Despite the fact that the synthesis and use of this molecule as a recognition element has already been described by the above-mentioned authors, this is - to the

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best of our knowledge - the first time that the use of BAP for synthesising a MIP under Precipitation Polymerisation protocol has been reported on.

The high complimentarity between the functional monomer and the template molecule enabled a very tight ratio of functional monomer to template molecule of only 2:1. Their most likely interaction is depicted in the following paper. Therefore, all of the functional monomer used should ideally interact with the template molecule. In this way, no random functional monomer would be incorporated on to the polymer, thus diminishing the non-specific interaction due to the typical excess derived from the use of commercially available functional monomers. Despite the reduction of the functional monomer, all the functionalities of the template molecule were perfectly coped and, once the MIP had formed, interaction of the target analyte with the polymer should be through up to six hydrogen bonds, thus improving the retention of the target analytes on to the polymer.

Apart from the complimentarity in hydrogen bonding of the template and functional monomer, the fact of using both non-polar solvents and a hydrophobic cross-linking agent during the polymerisation process enhances the affinity of the template molecule and functional monomer towards each other. This is because these are the only polar molecules present in the media. Therefore, a very effective hydrogen bond is expected to be established before the polymerisation process which, in turn, leads to an effective recognition of the polymer on to the target molecule once the polymer has been synthesised.

Another reason for selecting this particular approach when synthesising the MIP was that all barbiturates share the same core structure. Therefore, high cross-selectivity of the resultant MIP is expected to occur. This high selectivity can be explained by considering that retention of barbiturates is going to be through this core structure and, since differences between barbiturates are in their aliphatic moieties, no differences in retention of different barbiturates on the MIPs are expected to occur, apart from those arising from steric impediments, as was the case with the paraben report.

From the analytical point of view, the interest in detecting barbiturates is because these compounds are sometimes misused and this fact may have undesired consequences. Barbiturates act on the central nervous system and should only be used under medical prescription since their action is so strong that it can cause death. Another reason for controlling barbiturates is because, in some cases, these compounds are misused to provoke sensations such as psychotropic effects or to induce dizziness for illicit purposes, and these uses can also lead to the same fatal consequences. Therefore, the development of an easy and reliable method for detecting them will enormously facilitate their control. On the one hand, control of barbiturates would help to control the dosage of these compounds for people who suffer from anxiety or obsessive-compulsive disorders and need barbiturates on a daily basis [5], since a more customised and controlled prescription of the dose

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required would be possible. On the other hand, this protocol would also facilitate a more reliable method for detecting the presence of these compounds for any illicit purpose [6]. Under any of the above situations, control of such substances becomes of prime interest.

The method proposed in the following study involves the development of a MISPE protocol for selectively extracting barbiturates from human urine samples and their further determination using LC-UV. This is a very straight forward method for detecting the intake of barbiturates in the human body.

In this case, the results derived from this study have been submitted to Journal of Chromatography A for their publication.

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2.4.1 Molecularly imprinted polymers with high-fidelity binding sites for the selective extraction of barbiturates from human urine

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Molecularly imprinted polymers with high-fidelity binding sites for the selective extraction of barbiturates from human urine

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ABSTRACT

In this paper we describe the synthesis of a molecularly imprinted polymer (MIP) by precipitation polymerisation, with barbital as the template molecule, and the application of the barbital MIP as a molecularly selective sorbent in the solid-phase extraction (SPE) of barbiturates from human urine samples.
The MIP was synthesised by precipitation polymerisation using 2,6-*bis*-acrylamidopyridine as the functional monomer and DVB-80 as the cross-linking agent. The spherical MIP particles produced were 4.2 ± 0.4 μm and 4.8 ± 0.4 μm in diameter for the MIP and a non-imprinted polymer (NIP), respectively.
The particles were packed into a solid-phase extraction cartridge and employed as a novel sorbent in a molecularly imprinted solid-phase extraction (MISPE) protocol. The MIP showed high selectivity for the template molecule, barbital, a feature which can be ascribed to the high-fidelity binding sites present in the MIP which arose from the use of 2,6-*bis*-acrylamidopyridine as the functional monomer. However, the MIP also displayed useful cross-selectivity for other barbiturates besides barbital. In the MISPE studies, the MIP was observed to outperform a wide range of commercially-available sorbents in the selective extraction of barbiturates from aqueous matrices. Due to the high selectivity realisable through MISPE, the MIP was applied successfully to a tandem cartridge, in order to remove the urea present in the urine sample.

1. Introduction

The detection of regulated substances has always been of prime interest in different fields, not only to detect illicit consumption on self-administration [1], but also for forensic purposes to prove that regulated substances have been used to commit crimes such as sexual assaults [2] and suicide [3] or abused as performance-enhancing drugs in sports [4,5], for example.

Regulated drugs of interest are usually present at very low concentration levels in samples, therefore the detection methods used for their quantification must typically be very sensitive. This is the reason why the mostly commonly used separation techniques in this area, such as liquid-chromatography (LC) or gas-chromatography (GC), are often coupled to mass spectrometry (MS) [2,4,6].

In the majority of the cases reported concerning the detection of regulated drugs, pre-treatment of the sample is required. The most widely used sample pre-treatment method is solid-phase extraction (SPE). SPE is used to preconcentrate the analytes present in the sample and aid in the removal of interferences. However, in conventional SPE protocols not only is the target of interest preconcentrated, but other compounds are often retained by the SPE sorbent too. One way in which the preconcentration of the sample can be made more selective is to use designer sorbents which show molecular selectivity for the analyte of interest; an important class of sorbents which fall into this category are molecularly imprinted polymers (MIPs). When MIPs are applied as SPE sorbents, the technique is termed molecularly imprinted solid-phase extraction (MISPE).

Over the last few years, MISPE protocols have been used widely to facilitate difficult chemical separations.

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This is because MIPs are very often not particularly difficult to synthesise or obtain and their use can lead to significant enhancements in the selective retention of target analytes, even when low concentrations of analytes are being extracted from complex matrices [7]. MISPE typically leads to cleaner samples and lower limits of detection.

MIPs have been used to extract many different substances from a variety of sample types [8,9], including herbicides from vegetables [10], non-steroidal anti-inflammatory drugs [11] from water, naphthalene sulphonates from river water [12], metabolites of illicit drugs from aqueous matrices at clinically relevant concentrations [13], and pharmaceuticals from urine [14].

When designing a MIP, rational selection of the functional monomer is mandatory in order for the MIP to have the desired molecular selectivity when in use. In the majority of published examples where MIPs have been synthesised, commercially available functional monomers are normally used since there is a wide range of monomers to select from. However, for some templates even better molecular recognition outcomes may be realisable through the synthesis and implementation of designer functional monomers which are tailor-made for the analyte of interest [15]. In this context, in the present study we synthesised a functional monomer which was tailor-made to complement the hydrogen bonding motif found in barbital, which was our analyte of interest. This functional monomer, which can interact with barbital though a triple hydrogen bonding array, offered the prospect of novel MIPs being synthesised and used in MISPE, where the MIPs have high-fidelity binding sites in place and correspondingly reduced levels of non-specific binding when used in MISPE [16].

Recently, Haginaka *et al*. described the synthesis and application of a MIP with size-exclusion properties (a RAM-MIP; RAM = restricted-access material) for the extraction of barbiturates from river water samples [17]. Here, the authors synthesised their MIP by multi-step swelling polymerisation, with cyclobarbital as template molecule and a commercially available monomer (4-vinylpyridine) as the functional monomer. The RAM-MIP material was packed into a column and connected on-line to a chromatographic system coupled to a mass spectrometer. This set-up enabled the quantification of amobarbital, cyclobarbital, phenobarbital and phenytoin from river water samples (50 mL sample volumes).

In the present work, not only did we target a designer functional monomer for barbital to enhance the molecular recognition profile of the MIP, but by performing the template polymerisations under precipitation polymerisation conditions we were keen to synthesise spherical polymer particulates (beads) in a single preparative step [18]. The latter objective potentially delivers polymer particles which ought to perform rather well when applied as sorbents in packed columns in flow-through applications, due to their low particle size and narrow particle size distributions. A further objective was to use the novel materials for the selective extraction of barbital and related compounds from human urine samples to demonstrate their value in a challenging and practically useful chemical separation and analysis process.

2 Experimental

2.1 Reagents and standards

The compounds used to synthesise the functional monomer, 2,6-*bis*-acrylamidopyridine (BAP), were 2,6-diaminopyridine, acryloyl chloride and triethylamine (all from Aldrich, Steinheim, Germany); all were used as received.

The compounds used in the synthesis of the MIP and NIP were barbital (BAR; template molecule) divinylbenzene-80 (DVB-80; cross-linking agent) and AIBN (free radical initiator), both from Acros Organics (Geel, Belgium). Prior to use in polymerisations, DVB-80 was passed through a short column packed with neutral alumina, to remove polymerisation inhibitor, and AIBN was recrystallised from MeOH.

The solvents/porogens used for the polymerisations were acetonitrile (ACN) and toluene (both were of analytical grade and supplied by Riedel-de-Haën, Seelze, Germany). The solvents used in the chromatographic evaluations and MISPE experiments were ACN, methanol (MeOH), dichloromethane (DCM), chloroform, ethyl acetate (EtOAc), acetic acid (HOAc) and isopropanol; all were of HPLC grade and were supplied by SDS (Peypin, France). Phosphoric acid (H_3PO_4) was from Merck (Darmstadt, Germany), and water was sourced from a Milli-Q purification system (Millipore, Molsheim, France).

The analytes used during the study were barbituric acid, BAR, phenobarbital (PHN), pentobarbital (PNT) and secbarbital (SEC), and were sourced from Sigma Aldrich (St. Louis, MO, USA). The chemical structures of the analytes are presented in Fig. 1. A stock solution (1000 mg L^{-1}) for each analyte was prepared in water:MeOH (1/1, v/v) and stored in a fridge when not in use; diluted solutions were daily prepared from the stock solution.

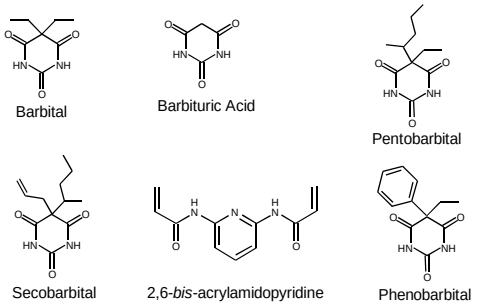

Fig. 1. Molecular structure of the barbiturates used in this study and the functional monomer.

To remove the urea present in the urine samples, a tandem cartridge system using a selection of commercially available sorbents was developed. In this regard, the sorbents tested were: Oasis® HLB (styrene-divinylbenzene-4-vinylpyrrolidone copolymer, 200mg) from Waters (Milford, MA, USA); Strata™ SDB-L (styrene–divinylbenzene copolymer, 200 mg), from Phenomenex (Torrance, CA, USA); Bond Elute LRC® (C_{18}, 200 mg) from Varian (Harbor City, CA, USA).

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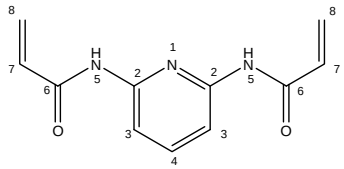
2.2 Synthesis of the functional monomer

2,6-*bis*-Acrylamidopyridine was synthesised according to a method reported by Yano *et. al.* [19]. Briefly, 2,6- diaminopyridine (5.46 g, 50 mmol) was suspended in chloroform (150 mL) and acryloyl chloride (10.90 g, 120mmol) added dropwise to the solution in the presence of triethylamine (12.1 g, 120 mmol) at ice-bath temperature and left to stir overnight at 0 ºC. After this time, the volatiles were removed under reduced pressure and the residue was dissolved in MeOH (100 mL). 50 mL of the methanol solution was then added slowly to deionised water (800 mL) with stirring. The product which precipitated was collected by vacuum filtration, washed with deionised water and then dried *in vacuo* at 40 ^{o}C to constant mass.

The characterisation data for the polymerisable template were as follows; for ^{1}H NMR (500 MHz, acetone) δ_H = 9.34 ppm (s (broad), 2 H, H_5); 8.01 ppm (d, 2 H, $J_{H3\text{-}H4}$ = 7.5 Hz, H_3); 7.78 ppm (t, 1 H, $J_{H4\text{-}H3}$ = 7.5 Hz, H_4); 6.59 ppm (dd, 2 H, $J_{H7\text{-}H8\ Cis}$ = 10 Hz, $J_{H7\text{-}H8\ Trans}$ = 17 Hz, H_7); 6.40 ppm (dd, 2 H, $J_{H8\ Trans\text{-}H8\ Cis}$ = 2 Hz, $J_{H8\ Trans\text{-}H7}$ = 17 Hz, $H_{8\ Trans}$); 5.76 ppm (dd, 2 H, $J_{H8\ Cis\text{-}H8\ Trans}$ = 2 Hz, $J_{H8\ Cis\text{-}H7}$ = 10 Hz, $H_{8\ Cis}$). For ^{13}C NMR (125 MHz) δ_H = 163.5 ppm (C_6); 150.7 ppm (C_4); 140.0 ppm (C_2); 131.5 ppm (C_8), 127.2 ppm (C_7); 109.3 ppm (C_3). ^{13}C NMR spectra were unequivocally assigned by means of HSQC spectra.



The MS the mass calculated for the PT ($C_{11}H_{11}O_2N_3$) was 217, which was found 216 for ESI (-) and 218 for ESI (+) . The elemental analysis composition was 52.95% C, 6,19% H and 15.98% O. The FT-IR data (KBr, cm^{-1}) 3490, 3243, 1682, 1626, 1586, 1536, 1448, 1407, 1326, 1290, 1243, 1204, 1151, 1069, 1012, 982, 956, 909, 800, 731, 621, 456. The melting point of the functional monomer could not be obtained due to polymerisation during the measurement.

2.3 Synthesis and characterisation of the molecularly imprinted polymer

The barbital MIP was synthesised by precipitation polymerisation using a non-covalent molecular imprinting approach. BAR (0.095 g, 1.3 mmol), BAP (0.209 g, 2.6 mmol), DVB-80 (1.3405 g, 26.5 mmol) and AIBN (0.112 g; 3 mol% relative to the number of moles of polymerisable double bonds) were dissolved in a 3:1 (v/v) mixture of ACN:toluene (40 mL; 4% w/v of total monomer w.r.t. solvent) in a 100 mL polypropylene bottle. The pre-polymerisation mixture was deoxygenated by sparging with oxygen-free N_2 for 5 min in an ice bath, the bottle sealed under a N_2 atmosphere and the mixture left to polymerise while rotating the bottle slowly about its long axis on a low-profile roller (Stovall, Greensboro, NC) in a temperature-controllable incubator (Stuart Scientific, Surrey, UK). The temperature was ramped from room temperature to 60 ºC over a time period of approximately two hours and was held at this temperature for a further 46 h. Once the reaction was complete, the particles were recovered by vacuum filtration on a nylon membrane filter prior to overnight Soxhlet extraction with methanol to remove template and unreacted starting materials and the polymeric yield was of 53%.

A non–imprinted control polymer (NIP) was synthesised in a fashion analogous to that used for the MIP, but without the addition of the template molecule and, in this case, the polymeric yield was of 78%.

All of the particles obtained were imaged by SEM using a JEOL JM-6400 Scanning Electron Microscope (Peabody, MA). The particle size distributions were measured using Image J Launcher software [18].

The specific surface areas of the MIP and NIP were measured by nitrogen sorption porosimetry using an ASAP 2020 Accelerated Surface Area and Porosimetry Analyzer (Micromeritics Instrument Corporation, Norcross, GA) followed by application of BET theory.

2.4 Chromatographic analysis

For the chromatographic evaluation of the polymers, two stainless steel LC columns (50 mm x 4.6 mm ID) were slurry packed with ~ 0.5 g of both MIP and NIP particles using an air-driven, fluid pump (Alltech, Model 1666) with acetone as the slurrying and packing solvent. These columns were applied as LC stationary phases to confirm the molecular recognition character of the MIP. Twenty microliters μL aliquots of 0.5 M standard solutions of barbituric acid, BAR, PHN, PNT and SEC, as well as acetone (void marker), were injected sequentially onto either the MIP or the NIP. The mobile phase was ACN:H_2O (25:75) (adjusted to pH ~ 3 with H_3PO_4) at a flow rate of 1 mL min^{-1}, using a UV detection set at 210 nm. From the data obtained through the various LC experiments, the retention factors and imprinting factor (IFs) were calculated.

When the MIP was used for MISPE studies, a commercially available LC column (Kromasil 5 µm, 100 Å C_{18}, 250 mm x 4.6 mm from Teknokroma.) was used to separate the analytes. A gradient elution profile with ACN and acidified water (adjusted to pH ~ 3 with H_3PO_4) was applied. The gradient started with 20% ACN and was increased to 60% in 4 minutes. From minute 4 to minute 8 the gradient was increased again from 60% to 70% ACN and reached 100% ACN at minute 10. From minute 10 to 13 the ACN content was decreased again to the initial conditions (20% ACN). The column temperature was maintained at 30 ºC and the UV detector set to monitor at 210 nm.

For all the chromatographic studies, the chromatographic instrument used was a Shimadzu LC-10AD binary liquid chromatograph equipped with a DGU-14A degasser, an injection loop of 20 µL, a CTO-10A oven and an SPD-10A UV detector. All of the LC modules were from Shimadzu (Tokyo, Japan).

2.5 MISPE conditions

For the MISPE analyses, ~ 60 mg of both MIP and NIP polymers were packed into two empty 6 mL polyethylene cartridges (Symta, Madrid, Spain). Given the particle sizes of the MIP and NIP, the upper frit was of polyethylene with a pore size of 10 µm (Applied Separations, Allentown, PA, USA) whereas the bottom frit was a metallic frit with a pore size of 2 µm (Supelco, Bellefonte, PA, USA). The cartridges were connected to a 12-port SPE manifold (Teknokroma, Barcelona, Spain) which was connected, in turn, to a vacuum pump.

When analysing urine samples, the urea was removed from the samples prior to the MISPE protocol.

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This involved the percolation of 2 mL of human urine (previously adjusted to pH ~ 3 with H_3PO_4 and filtered using an 0.22 µm pore size nylon) through an Oasis HLB cartridge; the cartridge was then flushed with 15 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4) and eluted with EtOAc (5 mL). The eluted fraction was evaporated under a stream of N_2 and reconstituted in 1 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4) prior to percolation through the MIP and NIP cartridges.

The MISPE protocol involved activation of the MIP cartridge with 15 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4), loading of the cartridge with the sample at pH ~ 3 (adjusted with H_3PO_4), cleaning of the sample using 1 mL of EtOAc and elution from the cartridge with 5 mL of MeOH (containing 1% HOAc).

The eluted fraction was evaporated to dryness under a stream of N_2 before reconstitution in 1 mL of acidified water (adjusted to pH ~ 3 with H_3PO_4). The reconstituted sample was then injected onto the chromatographic system.

Both before and after the percolation of the cleaning solvent, the cartridges were left to dry as completely as possible under high vacuum.

3 Results and discussion

3.1 Synthesis and evaluation of the MIP

In this study we decided to extract barbiturates from highly complex matrices using a MIP and synthesise this polymer by precipitation polymerisation protocol because of the advantages that this protocol brings over other existing ones [21].

Since all barbiturates have the same core structure, we synthesised a functional monomer according to the functionalities in their core structure.

This core structure is particularly interesting because is perfectly symmetrical and the functional groups are disposed alternatively in β position as can be seen in Fig. 1. Another interesting feature of this core structure is that has an acceptor-donor-acceptor (ADA) pattern in those functional groups so, the better the functional monomer matches this pattern, the higher the interaction of the MIP obtained with the target analytes [22]. Therefore, a good candidate which matches all these requirements is 2,6-*bis*-acrylamidopiridine (BAP) (Fig. 1), a functional monomer which has already been used in imprinting technology [23].

Having a closer look at the expected interaction of the functional monomer and the template molecule, since this functional monomer is able to establish up to three hydrogen bond per equivalent with the target molecule, using only a ratio of template molecule:functional monomer of 1:2, all of the functionalities in the core structure of barbiturates are coped. This ratio also brings the possibility of formation of up to 6 hydrogen bonds between the template molecule and the functional monomer, as shown in Fig. 2. This highly efficient interaction brings more defined binding sites once the MIP is formed and also diminishes the non-specific interactions on the MIP. Those non-selective interaction arise as a consequence of the normally used excess of functional monomer:template molecule when not tailorly synthesised functional monomer to the template molecule are used in the synthesis of the MIPs.

Once both the MIP and the NIP were obtained, the polymers were SEM imaged and, as can be seen in Fig. 3, fairly monodisperse and spherical particles were obtained. The size of the particle obtained were, in the case of the MIP and the NIP of 4.2 ± 0.4 µm and 4.8 ± 0.4 µm, respectively. Moreover, we also measured their surface area and both polymers obtained were macroporous displaying a high and very similar surface area with values of 685 ± 15 $m^2 g^{-1}$ and 654 ± 15 $m^2 g^{-1}$ for the MIP and the NIP, respectively.

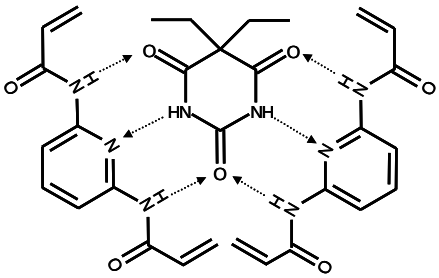

Fig. 2. Expected interaction of the template and the functional monomer and direction of the H bonds established.

Afterwards we moved to check the feasibility of the MIP for retaining the target analyte and related compounds. To do that, we checked the imprinting factor (IF) for the different compounds under study and the values obtained were of 8.64 for barbituric acid, 5.13 for BAR, 4.28 for PNT, 3.12 for PHN and 2.93 for SEC. These high IF values obtained for each compound demonstrate that retention of the compounds onto the MIP was actually through selective interactions. It is also well worth to note that, as the moieties on the core structure of the different barbiturates increased, its retention on the MIP decreased. This might be attributed to steric interactions of the larger molecules within the cavity left by the template molecule in the MIP. Those results are in good agreement with similar results recently reported by Haginaka *et. al*. [17], where the MIP obtained displayed higher affinity towards the template molecule and, as the moieties pendant from the core structure increased, retention of those compounds on the polymer decreased.

At this point, it is well worth to note that, despite barbituric acid was included when checking the IF values, since this compound does not have therapeutic effect, it was omitted when extracting real samples.

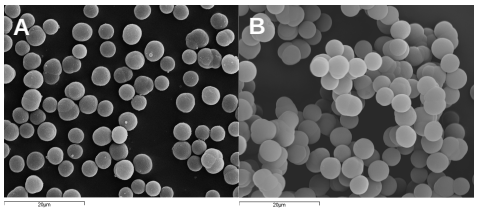

Fig. 3. SEM photographs of the MIP (A) and NIP (B).

3.2 MISPE experiments

For MISPE experiments, as loading solvents, 5 mL of several solvents such as acidified water (adjusted to pH ~ 3 with H_3PO_4), ACN, MeOH, isopropanol and DCM spiked at 0.5 mg L^{-1} of each barbiturate were percolated through both MIP and NIP, respectively. However, water at basic pH was not included in the loading study since compounds under study may be hydrolysed under basic

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conditions. For ACN and MeOH retention of the compounds under study was almost null (< 5%) whereas when using either DCM or isopropanol retention of those compounds were lower than 40% and 75% respectively. In the case of water, a complete retention for all the compounds was achieved (>99%) so this solvent water was eventually chosen as loading solvent.

From data obtained in the loading step, MeOH was chosen as eluting solvent since was the one with the lowest retention of the analytes on the MIP when this solvent was used as loading solvent. However, in order to reduce the volume of MeOH used in this step, addition of an organic modifier, in this case acetic acid (HOAc), was studied. Finally, the best volume and composition for eluting the compounds from the MIP was set at 5 mL of MeOH containing 1% (v/v) of HOAc since all the barbiturates were completely removed from the MIP (recoveries > 99%), whereas 5 mL of MeOH alone were not enough to remove all the compounds retained on the MIP.

In any MISPE protocol, but especially for those using water matrices, a cleaning step is highly desirable in order to remove as many as possible of the non-specifically retained compounds from the MIP, thus obtaining a cleaner extract. Moreover, to asses the selectivity of the active sites left on the MIP, a comparison between the MIP and the NIP was performed. To this end 1 mL of toluene or 1 mL of EtOAc were used to perform the clean-up step. In the case of toluene, after eluting both sorbents there was no difference between the MIP and the NIP, so toluene was not able to discern the selective interaction on the MIP from the non-selective ones on the NIP. However, as shown in Fig. 4, percolation of 1 mL of EtOAc had a dramatically different effect for the MIP or the NIP. In the NIP, 1 mL of EtOAc was enough to remove over 80% of each barbiturate retained on the NIP whereas in the MIP the removed fraction was less than 30% for BAR, PHN and PNT and 42% for SEC. Under this perspective, we decided to use 1 mL of EtOAc as cleaning solvent.

This behaviour further evidences the selective interaction of barbiturates on the synthesised MIP, supporting the IF values encountered during the LC evaluation of the polymers.

Once the whole MISPE was established, we also checked the maximum volume of sample able to be percolated through the MIP. Under the optimal conditions, recoveries obtained after percolation of 25 mL of ultra-pure acidified water were the same as those reported previously whereas when higher volumes were attempted, for instance 50 mL, recoveries were lower than 50% for each barbiturate.

3.3 MISPE of human urine

Once the good performance of the MIP was proven, we moved to extract barbiturates form urine samples. The reason to use this matrix was because urine enables a proper monitoring of the intake of drugs since is one of the main routes of excretion in the body.

Since, due to the high complexity of real samples, the recoveries of the analytes from real samples are always lower than those obtained from standard solutions used during the optimization of the method, we started using 5 mL of human urine instead of the maximum volume achieved when using ultra-pure water.

Surprisingly, despite the high affinity displayed by the

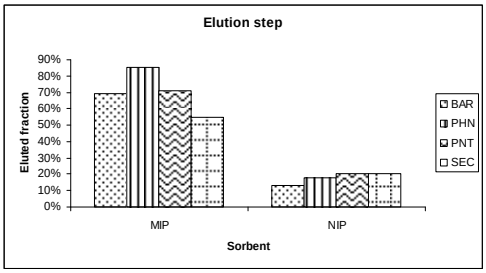

Fig. 4. Recoveries obtained for the MIP and the NIP after the MISPE protocol. Both sorbents were loaded with 5 mL of acidified water spiked at 5 mg L^{-1} of each barbiturate, cleaned-up with 1 mL of EtOAc and eluted with 5 mL of MeOH (1% HOAc).

MIP towards the barbiturates, no retention was observed when extracting 5 mL of human urine. Therefore, we decreased the volume of sample to 2 mL and, in this case, recoveries for all the barbiturates under study were below 15% after the MISPE protocol. Under this perspective, we presumed that the low retention of barbiturates onto the MIP could probably be due to the high urea concentration in the sample.

Since the functional monomer used in the synthesis of the MIP is a urea based monomer, the interaction of urea to this monomer is expected to be very high thus preventing the barbiturates present in the sample to interact properly with the MIP.

However, since our target was to extract barbiturates from human urine samples and our hypothesis was that the urea present in this sample prevented barbiturates to be retained on the MIP, we decided to establish a tandem system using a commercial sorbent in order to confirm this hypothesis and remove the urea present in the sample.

To do that, we tested three conventional SPE sorbents for retaining barbiturates while enabling a proper removal of the urea present in the sample.

We percolated 5 mL of acidified water spiked at 2 mg L^{-1} through a hydrophobic C_{18} sorbent (Bond Elut LCR), a styrene-divinylbenzene copolymer (Strata SDB-L) and a divinylbenzene-4-vinylpirrolidone copolymer (Oasis HLB). From this comparison, both C_{18} and styrene-divinylbenzene cartridge were discarded since losses up to 80% and 35%, respectively, during the loading step were observed. Oasis HLB was eventually selected because this sorbent could stand the loading and flushing of the sample and also the barbiturates retained on this sorbent could be eluted using 5 mL of EtOAc.

For urine sample, the Oasis HLB cartridge was loaded with 2 mL of human urine spiked at 5 mg L^{-1} of each barbiturate and flushed with 15 mL of acidified water to remove the urea present while keeping all the compounds of interest retained on Oasis. This sorbent was further eluted with 5 mL of EtOAc since this solvent was strong enough to remove the barbiturates retained on the cartridge. This eluted fraction from Oasis HLB was evaporated to dryness under N_2 stream and reconstituted in 1 mL of acidified water prior its percolation through the MIP. Once the MIP was loaded with this fraction, a clean-up step using 1mL of EtOAc was performed and eluted with 5 mL of MeOH (1% HOAc). This methanolic fraction was also evaporated to dryness under N_2 stream and quantified by LC.

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Recoveries from this tandem system were of 40%, 65%, 47% and 35% for BAR, PHN, PNT and SEC (RSD < 7.8%, n = 3), respectively.

Fig. 5 shows the chromatogram obtained after the extraction of 2 mL of human urine spiked at 5 mg L^{-1} after the MISPE protocol when using the tandem Oasis HLB-MIP in the extraction process. As can be seen in this figure, removal of urea was the key factor for the proper retention and further quantification of barbiturates onto the MIP.

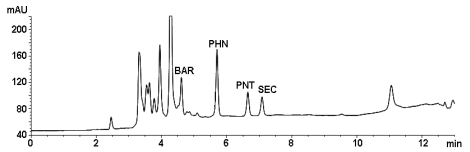

Fig. 5. Chromatogram obtained after the MISPE protocol of 2 mL of human uriune spiked with 5 mg L^{-1} of each barbiturate after the tandem system with a commercial sorbent.

Once the feasibility of the tandem system studied, we moved to validate the method in the range from 0.5 to 25 mg L^{-1} for each barbiturate which includes the level of barbiturates normally found in urine samples, as reported by Jiang *et. al.* [22] when determining barbiturates by capillary-electrophoresis means. The linearity was established by spiking 2 mL of human between 0.5 and 25 mg L^{-1} of each barbiturate and, after the tandem protocol, the R^2 value for the linearity of the method was above 0.996 for any of the barbiturates under study. The intraday repeatability was set using four consecutives replicates of 2 mL of human urine spiked at 1 mg L^{-1} and the values for this parameter were of 8.8, 8.1, 4.4 and 3.6% for BAR, PHN, PNT and SEC, respectively. Reproducibility between days was also tested under the same conditions during four different days and the values obtained were of 10.5, 11.2, 6.7 and 5.8% for all barbiturates under study and in the same order as above. The LOD was set using a signal to noise ratio of 3 and the values were below 0.2 mg L^{-1} in any case.

Therefore, the tandem system developed herein enabled the proper quantification of several barbiturates at clinically relevant concentrations from human urine samples by using a MIP.

4. Conclusions

A MIP using a tailor-made functional monomer according to the structure of barbital, the template molecule used, and obtained by precipitation polymerisation has successfully been synthesised in a good polymerisation yield and with high surface area.

The high-fidelity of the functional monomer to the template molecule enabled a high recovery not only for the template molecule but also for closely related compounds, showing a useful cross-selectivity of the MIP for barbiturates.

When applying the MIP to real samples a MISPE protocol was developed which included a tandem system with a commercial sorbent. This tandem system was necessary to remove the urea present in the sample because, since the functional monomer used in the synthesis is a urea based monomer, the presence of urea in the sample highly interferes, or even prevents, the rebinding of the target analytes to the polymer.

The use of a tandem system proved to be a very good alternative for discarding undesired compounds whose might be present in the sample and difficult the extraction of the compound(s) of interest.

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2.4.2 Discussion of Results

A MIP for the selective extraction of barbiturates using a self-designed functional monomer and obtained under Precipitation Polymerisation has been synthesised and successfully applied to extract these compounds from human urine samples.

As pointed out in the introduction to this chapter, the functional monomer chosen perfectly matched the functionalities in the core structures of barbiturates. This high complimentarity enabled a very tight ratio of template:functional monomer of 1:2 to be used that, in turn, also diminished the non-selective interactions due to the random incorporation of the normally used excess of functional monomer on the polymer.

From results obtained in the experimental part when comparing the ability of the MIP and the NIP to selectively retain the target compounds, the MIP showed far higher retention of these analytes than the control polymer. This means that the functional monomers used during the synthesis of the MIP were efficiently arranged along the template molecule, providing a good imprinting effect on the polymer and enabling an excellent recognition of the MIP towards these compounds. In contrast, when the same functional monomers are left to polymerise without the presence of the template molecule, these monomers are randomly distributed within the matrix of the polymer, displaying the same selectivity against the target molecule as to any other molecule present in the sample.

Another interesting result from the chromatographic evaluation of the MIP using related compounds to the template molecule is the lower retention of the different barbiturates under study on the MIP as the pendant moieties from their core structure increased. This can be explained because barbital was used as the template molecule and this compound has shorter pendant moieties. Therefore, the larger the pendant moieties on the core structure, the stronger the steric impediments and the poorer the retention of these compounds on the MIP, since their core structure can not be properly accommodated within the active site.

This result is in good agreement with the previously reported study in point 2.3 in which parabens were the molecules to be extracted with a MIP obtained using methyl paraben as the target molecule. In that case, retention for ethyl, buthyl and benzyl paraben decreased as the moieties of the core structure increased.

However, this poorer recognition towards the barbiturates does not mean that the MIP obtained did not retain these barbiturates at all. When comparing the retention of these molecules on the MIP with their retention on the NIP, a far higher recovery for barbiturates was obtained on the MIP than with the NIP.

Another outstanding result obtained during the evaluation of the MIP was the fact that, when comparing the performance of the MIP obtained with a full range of

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conventional SPE sorbents representative of the most widely used in SPE experiments, the MIP outperformed the conventional ones in all of the cases studied. Therefore, the use of the present MISPE technique for the selective extraction of barbiturates would improve their quantification by LC, since the most widely used sample pre-treatment for quantifying those compounds involves SPE with conventional sorbents [7,8].

To this end, the sorbents of choice were a hydrophobic sorbent (C_{18}, Bond Elute LRC), a polymeric sorbent for retention mainly by π-π interactions (styrene-divinylbenzene, Strata SDB-L) and a polymeric sorbent modified with 4-vinylpirrolidone (Oasis® HLB). As expected, neither C_{18} or styrene-divinylbenzene sorbents could even retain analytes during the loading of the cartridge since important losses during this step were already observed, due to their polar characteristic. Because of this, both sorbents were excluded from the study. For Oasis® HLB, all the analytes could be retained but, as expected, this sorbent, unlike the synthesised MIP, could not withstand the clean-up step because the retention of barbiturates on it was through non-selective interactions. Therefore, the use of the synthesised MIP makes it possible to perform more highly selective extractions and obtain cleaner extracts than using any of the commercially available sorbents.

These results further support the enhanced affinity of the designed MIP towards the analytes of interest. On the one hand, when comparing the MIP with the apolar sorbents used in the study, even though over 85% (w/w) of the MIP was of DVB (so retention of compounds could only be through either hydrophobic or π-π interactions), the presence of BAP enabled the selective retention of the target analytes. On the other hand, when comparing the MIP with the polar sorbents used in the study, although the content of pendant polar groups from commercial sorbents is higher than those on the MIP and since the group in these sorbents are not spatially arranged to match the structure of barbiturates, this sorbent could not withstand the washing step.

Even though barbiturates are normally extracted using commercially-available sorbents [8,9], the results obtained in this study clearly show that none of the commercially available sorbents can extract barbiturates more selectively from aqueous samples than the synthesised MIP. This is the differential value that this MIP has over the rest of the commercially available sorbents.

After all of the excellent results obtained during the evaluation of the MIP, we felt pretty confident to attempt studying real samples. Unfortunately, when attempting analysis of barbiturates in 2 mL of human urine, recovery of these compounds compared to the recovery obtained in ultra-pure water was very poor (~ 10% for pentobarbital and secbarbital) and even null for barbital and phenobarbital. We looked back at the matrix and hypothesised that the possible cause for the low recognition of barbiturates on the MIP was due to the high concentration of urea present in the sample. Since the functional monomer used is a urea-based

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monomer, the high concentration of urea could prevent barbiturates from being retained on the polymer, thus leading to the poorer recognition values obtained.

To prove this, the idea was to use a sorbent which retained the target analytes, flush it with plenty of water and recover the target analyte free from most of the urea present in the original sample. The fact of using water for removing the urea present in the sample was because urea is a small and highly polar compound so is expected to be easily removed with water while the rest of analytes be still retained on the cartridge. Afterwards, elution of those analytes with an organic solvent and further percolation of this extract through the MIP would achieve the high selectivity displayed by this sorbent.

This solution proved to be effective in removing the urea from the sample. After this tandem system, barbiturates present in urine samples were easily extracted and quantified by LC-UV.

Due to major concerns about controlled substances in general and barbiturates in particular, another study was recently published that described the development of a MISPE protocol for the detection of barbiturates [9]. This MIP was also obtained by PP but used commercially available functional monomers instead. When this kind of functional monomer was used, the retention of the analytes on to the MIP or the NIP is not as high as the one reported in the present study. This is despite the fact that the MIP synthesised in that paper enabled the selective retention of several barbiturates from river water samples. In this case the authors synthesised a restricted access material MIP (RAM-MIP) for excluding large molecules, such as soluble oligomers of humic acids in river water, to make it difficult for the target analyte to be properly retained on to the MIP.

After all, when it is feasible, the synthesis of the functional monomer according the functionalities of the target molecule has proven to be a good way for imparting the highest affinity to the MIP.

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2.4.3 Bibliography

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3. Conclusions

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The conclusions derived from the experimental results obtained in the present thesis are as follows:

1. Comparing the different synthetic protocols used in this thesis, Traditional Polymerization is the most straightforward technique for synthesizing molecularly imprinted polymers (MIPs) due to the simplicity of the synthetic protocol.

2. Different MIPs using target compounds with different polar characteristics have been successfully synthesised. In the case of highly polar compounds, such as amoxicillin and cephalexine (β-lactamic antibiotics), the derivatization of these molecules has enabled to synthesize two MIPs for the selective extraction of these compounds from aqueous samples.

3. When feasible, Precipitation Polymerization is a good alternative to overcome the main drawbacks in the synthesis of MIPs by Traditional Polymerization. This is due to its higher polymeric yields, the uniformity of particle size and shape, and the reproducibility of the particles obtained from the MIPs synthesized using either carbamazepine, an analogue of methylparaben or barbital as template molecules.

4. The MIP using carbamazepine as template molecule and obtained under Precipitation Polymerization exhibited higher breakthrough volume than the MIP obtained by Traditional Polymerization.

5. The inclusion of a sacrificial spacer in the semi-covalent approach for synthesizing MIPs under Precipitation Polymerization has proven to be feasible, as demonstrated by the MIP obtained using an analogue of methylparaben as the template molecule.

6. The semi-covalent approach delivers more defined active sites for the target analyte within the MIP, as demonstrated by the different recovery values obtained on a MIP and on its control polymer for a series of analogues of methylparaben (the target molecule). However, the single active point within the cavity was not enough to achieve high recoveries of the analytes of interest.

7. The design of the functional monomer according to the functionalities of the target analyte has proven to be a good way of obtaining high affinity for the intended MIP. This result can be confirmed by comparing the recoveries of the target analyte obtained on the MIP with those on the NIP. When using the designed functional monomer for the synthesis of a MIP targeted for barbital, the difference in recoveries obtained on the MIP to those obtained on the NIP were significantly higher than with any of the other MIPs synthesized using commercial functional monomers.

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8. As can be seen from all the studies performed in the present thesis, water-based matrices such as human urine, river water or effluent water from sewage treatment plants can be used directly to perform MISPE.

9. Some of the MIPs synthesised showed low breakthrough volume for the target analyte, as was the case of the MIPs using either amoxicillin or cephalexine as the template molecules. Some other MIPs, such as the MIP obtained using carbamazepine as template molecule and Precipitation Polymerisation or the MIP obtained using buthyl paraben as template molecule and obtained under Traditional Polymerisation, enabled a high volume of the sample to be extracted.

10. When aiming to detect low concentration levels of the analytes of interest using a MIP with low capacity, the use of a tandem system with a high-capacity sorbent is a good alternative, as proven by the extraction of cephalexine from river samples.

11. A tandem system using a commercial sorbent is also a very useful way to remove the urea present in human urine samples. Urea disrupts and prevents the retention of barbiturates on to the MIP synthesised using a urea-based functional monomer.

12. Since MIPs can be loaded with organic solvents, the conventional SPE sorbent can be eluted with an organic solvent and this fraction can be directly percolated through the MIP when using a tandem system, as was the case with the determination of cephalexine in river samples.

13. The cross-selectivity displayed by all of the MIPs obtained in this thesis enabled the selective extraction of not only the target analyte but closely related compounds as well.

14. MIPs are a good option to perform selective extractions from highly complex matrices and, considering all the new formats and applications currently being developed, there is still a long way to go for exploiting the advantages that these sorbents offer.

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Annex

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Annex: Publications derived from the present thesis.

- A. Beltran, E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, "Synthesis and application of a carbamazepine-imprinted polymer for solid-phase extraction from urine and wastewater", Anal. Chim. Acta 597 (2007) 6.

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- A. Beltran, R.M. Marcé, P.A.G. Cormack, F. Borrull, "Synthesis by precipitation polymerisation of molecularly imprinted polymer microspheres for the selective extraction of carbamazepine and oxcarbazepine from human urine", J. Chromatogr. A 1216 (2009) 2248.

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- A. Beltran, R.M. Marcé, P.A.G. Cormack, F. Borrull, "Molecularly imprinted polymers with high-fidelity binding sites for the selective extraction of barbiturates from human urine", J. Chromatogr. A (2010) submitted.

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