3 Molecularly Imprinted Polymers for Sample Preparation

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3.1 Scope and Interest

The sample treatment/preparation steps involve any operation and manipulation of the samples prior to instrumental determination of the target compounds [1–3]. This is a critical step in the analytical process because the concentration of target analytes in the samples is frequently too low for quantification. Thus, the first step of the preparation protocol involves the exhaustive extraction/concentration of the analytes from the matrix. Commonly, the initial step is nonselective and the obtained extracts require subsequent purification before final instrumental determination. Purification or clean-up treatments are usually carried out off-line, which negatively affects throughput and analysis cost in terms of both time and reagent consumption, makes the procedures susceptible to contamination and degradation of the analytes, and often results in the generation of relatively large amounts of waste. Appropriate selection and optimisation of the sample preparation procedure is a key aspect within the analytical process which can greatly affect the reliability and the accuracy of the results [1, 4].

Environmentally friendly and more efficient analytical methodologies consistent with the principles of green chemistry are currently being developed. This means that the use of highly toxic reagents is minimised, the time and energy consumption of processes is reduced, and a broad spectrum of target analytes can be determined in a single analytical run. The selection of an extraction technique is made on the basis of several factors, among which the nature of the matrix and the analytes are of primary importance. The speed of extraction, complexity of the instrumentation, and simplicity, flexibility and robustness of the method are also crucial. Moreover, selectivity of the extraction step is often a key factor for target-compound analysis [5].

Improvements in the efficiency of sorbents make the extraction technique suitable for the determination of analytes with different chemical structures and polarities. Currently, research is mainly focused on the development of:
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- Membranes to be placed at the interface of the two solvents in liquid-liquid extraction;
- Hollow fibre polymers for hollow fibre microextraction;
- Materials for sorption-based extraction, such as solid-phase extraction (SPE), microextraction in a packed syringe, dispersive solid-phase extraction (d-SPE) on which quick, easy, cheap, robust, effective and safe methods are based, and solid-phase microextraction (SPME);
- Coatings for stir-bar-sorptive extraction (SBSE); and
- Small particle sorbents to be packed into an empty column, SPE cartridge or syringe for matrix solid-phase dispersion (MSPD).

Novel sorbents allow higher loading capacities and higher efficiency for the retention of highly polar analytes from aqueous matrices. Additionally, improved selectivity during the retention process may contribute to the simplification of the subsequent clean-up and detection steps [6, 7]. Molecularly imprinted polymers (MIP) can be useful as efficient and selective sorbents in all the above-mentioned methods (Figure 3.1). As an example, MIP have been shown to increase the selectivity of the SPME overcoming the need of previous fractionation and purification steps in the analysis of complex extracts, and minimising the risk of instrument contamination [8]. For an adequate performance of MIP as selective sorbents, the solvent used in the extraction step must have a polarity similar to that of the solvent used in the polymerisation process, in order to increase the number of interactions between the analyte and the specific binding sites [9].

SPE is perhaps the most widely used technique for the preconcentration and clean-up of analytes from fluids and aqueous samples. MIP particles for solid-phase extraction (MISPE) can be packed into a high performance liquid chromatography (HPLC) precolumn for an on-line work mode, minimising sample manipulation and reducing the loss of analytes compared with the off-line cartridges where the MIP particles are situated between two frits [10]. The extraction involves four steps:

- Conditioning: MIP particles are treated with adequate solvents to ‘activate’ the imprinted cavities, i.e., to ensure that the size and the chemical groups in each cavity are in the best condition to recognise the target analyte;
- Sample loading: the liquid phase containing the analyte enters into contact with the sorbent. The solvent should be chosen to maximise the analyte-MIP interactions;
- Clean-up: a washing step is carried out with a solvent that removes interfering compounds nonspecifically interacting with the MIP; and
- Elution: the analyte-MIP interactions are disrupted by means of a suitable solvent.
Figure 3.1 Applications of molecularly imprinted polymers (MIP) in sample preparation methods. SPE: solid-phase extraction; MEPS: microextraction packed sorbent; MSPD: matrix solid-phase dispersion; SPME: solid-phase microextraction; and SBSE: stir-bar sorptive extraction

The presence of nonselective binding sites in the MIP may lead to the coextraction of some matrix components that could interfere with the chromatographic determination of target compounds [7]. This drawback might be overcome by the introduction of a previous clean-up step using a nonimprinted polymer (NIP); this approach is called two-step MISPE. The sample is first loaded into the NIP-containing cartridge and thus analytes, and some matrix components, are nonspecifically retained. Then, target analytes and some interfering compounds are eluted during the washing steps, whereas the majority of the matrix compounds remain bound to the NIP; thus, a first clean-up of the sample extracts is achieved. Later, the obtained washing solution is transferred to the corresponding MIP for further clean-up [11].

The development of a new MIP, suitable for a specific template molecule, often requires a lot of time and work due to the complexity of the design, along with the many variables involved in the synthesis, template extraction and testing. Working in aqueous medium, as required for pharmaceutical, clinical and environmental applications, makes the task even more complicated. Water molecules compete with the template, making weaker or destroying noncovalent interactions (electrostatic, hydrogen and van der Waals bonds) with the functional monomer. Hydrophobic and ionic interactions, metal coordination and cyclodextrin complex formation are proving to be very promising to enhance template-functional monomer association in water [12, 13].
3.2 Synthesis of Molecularly Imprinted Polymers for Sample Preparation

An overview of the literature reveals that the main analytical applications of MIP are in separation [14, 15], as stationary phases during both HPLC and capillary electrochromatography (CEC), unifying the steps of sample clean-up and analyte detection, and decreasing the time of analysis [16]. The selective recognition property, in addition to the robustness and ease of preparation, of MIP have made them attractive for a wide range of applications in clean-up and sample preconcentration [7, 17, 18]. This step is very challenging in the analysis of complex samples. MIP can successfully replace other adsorbents due to their extraordinary features of affinity and selectivity.

Several variables of the imprinting process can affect the selectivity and binding capacity of MIP [7, 18]. Interactions among the template molecule, functional monomer and crosslinking agent, by means of hydrogen bonding and electrostatic and/or van der Waals forces, are required to create short-range molecular organisation at the receptor site. The polymer morphology and MIP selectivity are affected by the concentration and stoichiometry of the template/functional monomers. During polymerisation, phase separation is affected by the temperature and porogen used in the process, which determines the polymer morphology, porosity and accessibility of the binding site. It also influences the interactions between the functional groups and the template molecule. MIP selectivity and capacity also depend on the temperature sensitivity of the binding equilibrium between the functional monomers and the template [15].

3.2.1 Approaches to Molecularly Imprinted Polymer Preparation

MIP are mainly prepared using three approaches: covalent, noncovalent and semicovalent binding of the template and the functional monomers [19]. The last one renders a particular type of covalent polymer, in which the initial interaction between the template and the monomer is through a covalent linkage, strong and stable during the synthesis of the MIP, but the interaction involved in the subsequent recognition of the analyte is noncovalent [20]. Covalent molecular imprinting allows the best structural definition in the shape and size of the cavity generated by the analyte, but this technique is not very versatile as there are few monomers and analytes with functional groups suitable for forming such links. Conversely, more monomers can interact with the analyte through noncovalent bonds, which has enabled the application of molecular imprinting to a wide variety of templates. Generally, a template:monomer molar ratio of 1:4 is sufficient to achieve the objective. Excess free monomer after polymerisation may lead to the formation of nonspecific binding sites on the polymer, which may interfere in the extraction/concentration of the analyte and necessitates optimisation of the solutions for loading, washing and elution [18, 21]. Advantages
and disadvantages of covalent and noncovalent approaches are discussed in Chapter 2. Semicovalent polymers have several advantages similar to covalent polymers, such as high selectivity and more uniform distribution of binding sites in the matrix [22]. The main drawback results from steric differences between the covalent and noncovalent bonds, since a greater distance is required to form a noncovalent bond than a covalent one. In general, covalent MIP demonstrate greater effectiveness than noncovalent MIP when used as the stationary phase in chromatographic systems [23].

3.2.2 Optimisation of the Molecularly Imprinted Polymers Synthesis

The most investigated variables of the synthesis of MIP are the nature and amount of monomer, initiator, crosslinker and porogen, method of initiating polymerisation and polymerisation conditions. Several tools can be applied to select an optimal MIP formulation, as follows:

• Mini-MIP or combinatorial imprinting [24, 25];
• Chemometric methodology [26, 27]; and
• Molecular modelling or dynamic study of the interactions of the analyte with various monomers [28, 29].

Combinatorial imprinting relies on the systematic variation of the mixture composition used in the synthesis of MIP, employing an automatic dispensing system that pours and mixes small volumes of reagents in a series of vials. Following polymerisation and template extraction by repeated washing of the polymer, a method of ‘screening’ is applied, either sequentially or in parallel. Once the MIP with the best performance are identified, larger quantities of those polymers are synthesised for further evaluation. Due to the large number of mixtures to assess, they are usually prepared automatically [30]. The main drawback of this method is the difference between the behaviour of MIP under equilibrium conditions (characterisation and optimisation of analyte recognition) and nonequilibrium conditions (used in chromatographic systems or extraction techniques). This difference in performance makes extrapolation of the results difficult and hinders the simultaneous optimisation of the large number of variables involved in the possible combinations.

Chemometric methodology is based on the use of experimental designs which allow optimising a number of variables simultaneously [27, 31]. A multidimensional matrix, which includes the whole range of values of all the variables that can influence the operation of the polymer, is generated. Some representative MIP are selected, prepared and characterised. Doehlert design is a widespread chemometric tool used for optimisation of MIP formulations [32]. Multivariate analysis of the results leads to response surfaces suitable for predicting the optimal procedure to obtain the MIP [33–35].
Molecular modelling relies on the *in silico* evaluation of the bond strength between the analyte and the functional monomers, simulating the different experimental conditions employed during polymerisation [28, 29]. This information is used to synthesise the polymers that *a priori* should possess imprinted cavities of high affinity and stability. The experimental results generally confirm the value of the approach [36].

The screening procedures described above do not consider factors, such as polymerisation rate and template aggregation, that have been demonstrated both experimentally and computationally to affect the properties of the imprinted cavity [37]. Atomistic simulation of MIP polymerisation focuses on the interactions that are responsible for recognition within the imprinted cavity [38, 39]. The molecular modelling simulations have been combined with a kinetic gelation model in order to describe the polymerisation process. Specific hydrogen bonds found in the prepolymerisation complex can be maintained after polymerisation, supporting interest in the characterisation of the prepolymerisation interactions. Nevertheless, MIP simulation on an atomistic scale is computationally expensive because of the large number of atoms involved (from the monomers, analyte and solvents). Functional site heterogeneity, template aggregation and restructuring of the imprinted cavity are aspects of the molecular imprinting process that cannot be addressed at this level of detail, due to the limitation of computational power. The coarse-grained theory offers a better understanding of the imprinting process as a whole. Such models provide important information about the intricate balance between stoichiometry, equilibrium versus kinetics and MIP performance [40–43]. They can show the binding site heterogeneity both in terms of structure and functionality, missed by the atomistic simulations, and experimentally replicate measured trends for MIP such as imprinting effect, binding site distributions and separation factors.

### 3.2.3 Requirements of the Molecularly Imprinted Polymers for Sample Preparation

Many variables have to be assessed since they can influence the morphology, structure and performance of the polymers [9, 44]. Polymerisation generally follows a radical propagation mechanism and can be both thermally and photochemically induced [45]. Free radical polymerisation is performed under mild reaction conditions (e.g., temperature lower than 80 °C and atmospheric pressure) in bulk or in solution, and it is suitable for a wide range of functional groups and template structures. Photoinitiated polymerisation at low temperature decreases the kinetic energy of the prepolymerisation complex, increasing its stability and allowing greater binding capacity and specificity than thermally initiated polymerisation, which requires temperatures higher than 40 °C [9]. Although synthesis of MIP is usually performed using a single functional monomer, in certain cases a mixture of monomers is used with the aim of improving the selectivity of the subsequent interaction between the
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polymer and the analyte. As explained in earlier chapters of this book, selection of the functional monomers and optimisation of the template:functional monomers stoichiometry are critical [20, 46]. Common functional monomers are carboxylic acids (e.g., acrylic acid, methacrylic acid (MAA), vinylbenzoic acid), sulfonic acids (e.g., 2-acrylamido-2-methylpropane sulfonic acid), and heteroaromatic bases (e.g., vinylpyridine, vinylimidazole). MAA is the preferred monomer for interacting with basic compounds, whereas 4-vinylpyridine (4-VP) is preferred for acidic compounds. However, since both monomers can establish a strong hydrogen bond, they have been used for extracting either acidic or basic compounds.

The morphology of the polymer is determined by the properties of both the porogen and the crosslinker, as they exert a combined influence on the porosity, which must be sufficiently high in order to allow access of the analyte to the binding sites. In some cases, the porosity can be tuned by applying postpolymerisation treatments. The crosslinker is primarily responsible for the morphology and yield of the polymer matrix, stabilises the selective binding sites, confers mechanical and thermal stability to the polymer, and provides adequate porosity to ensure accessibility of the analyte to the cavities [47]. Although there are many different crosslinking agents to choose from, the most widely used are ethylene glycol dimethacrylate (EGDMA) and trimethylolpropane trimethacrylate (TRIM). The latter gives polymers with more rigidity, structural order and effective binding sites than EGDMA, and with a more uniform size and higher yield than divinylbenzene (DVB) [48]. For most functional monomers, the selective retention of the analytes in the polymer can be established by H-bonds or ionic interactions, depending on the solvent and pH of the solution being percolated [8]. The stability of the links between the monomers and the template is highly favoured by the use of apolar solvents with a low dielectric constant, such as toluene and chloroform. Protic solvents disrupt hydrogen bonds. Polar solvents, such as water or methanol (MeOH), are suitable when hydrophobic forces are involved in the complexation process [49].

To prevent template bleeding during sample preparation, the use of a close structural analogue of the target analyte (dummy template) can be advisable [9]. However, this can result in inferior molecular recognition ability compared with MIP prepared using the analyte as the template. This drawback can be minimised using an analogue whose shape and functionality are as similar as possible to those of the analyte, e.g., a stable isotope labelled compound [7]. Even if template leakage occurs, it would not affect the quantification of the analyte if an adequate detector is used. The use of an isotope-labelled compound as the dummy template can notably improve the reproducibility of the analysis, allowing precise and accurate quantification of the target analyte at an ultratrace concentration level [50].

The polymerisation method affects the morphology of the polymer and the recognition of the analyte [30, 51], and thus it has to be adapted to provide formats suitable for
each application of the MIP [7, 18]. The development of polymerisation strategies to synthesise MIP microspheres includes: (i) obtaining regular particles with adequate packaging features, and (ii) improvement of the contact of the analyte-containing sample with the imprinted cavities [18, 19].

3.2.3.1 Bulk Polymerisation

All the components are dissolved in a small volume of a suitable solvent, which can also serve as a porogen, and then the polymerisation is photochemically or thermally initiated. The insoluble polymer monolith is ground and sieved to obtain micrometric irregular particles and the template is removed [18]. The main drawback of this method comes from the partial destruction of the imprinted cavities during the grinding process, which in turn decreases the binding capacity of the MIP [52]. Nevertheless, it is a widespread, simple method that can be carried out under mild conditions (room temperature), and requires limited organic chemistry and no specialised equipment. Numerous MIP reported as home-made fibres for SPME [53] and SBSE [54], or selective adsorbents for SPE, are based on bulk polymerisation [55–63].

Since particle heterogeneity may be a problem for some applications of MIP, various other polymerisation methods have been developed for obtaining beads and microspheres of uniform size [19, 45].

3.2.3.2 Precipitation Polymerisation

This methodology is analogous to bulk polymerisation, but involves a much more dilute mixture of monomers, working with an excess of solvent. As a result, the polymer grows in the form of independent spherical nanoparticles, with a high yield and homogeneous distribution of the binding sites [7, 18, 64]. The particle size of the resultant MIP is very small (about 0.3 to 10 µm), and depends on the template nature and stirring conditions [65–68]. Several MIP obtained by this method have been evaluated for SPE applications, such as extraction of tebuconazole from biological and environmental samples [69], thiabendazole from fruits [70], or methamphetamine and ecstasy from urine [71]. Barbital imprinted particles (4 µm diameter) bearing 2,6-bis-acrylamidopyridine as the functional monomer were used as the sorbent of MISPE, and selectively extracted the template and other barbiturate compounds from human urine after the removal of urea [72]. Pérez-Moral and Mayes [51] reported MIP for propanolol prepared by precipitation polymerisation which provided better recognition than those synthesised applying bulk polymerisation, suspension or emulsion polymerisation in toluene or water.
3.2.3.3 Suspension Polymerisation

In this technique, a hydrophobic organic medium containing the monomers and another organic or aqueous dispersion medium, immiscible with the former, are mixed with the help of a dispersant (e.g., perfluorinated liquids). The mixture is stirred to form microdrops in which polymerisation takes place [18, 73, 74]. The size of the particles (usually in the range of 10−100 µm) can be controlled by the addition of a stabiliser (polyvinyl alcohol or an ionic liquid) and tuning the stirring rate [75, 76]. In contrast to bulk polymerisation, stabilisers/surfactants are added and the monomer/template concentration in the prepolymerisation solution is much lower [52]. Two disadvantages of the method include the disruption of the monomer-template complex, due to the presence of surfactants, and the remnant amounts of stabilisers in the polymer particles after extensive washing [52]. In a comparative study, MIP obtained by bulk polymerisation showed a higher capacity and selectivity, due to the larger pore sizes, than the same MIP prepared by suspension polymerisation [77]. Magnetic MIP beads of 40–200 µm synthesised via suspension polymerisation exhibited a rough, porous surface and provided good, selective extraction of β-sitosterol and its analogues from biological samples for sensitive detection by gas chromatography-mass spectrometry (GC-MS) [78]. A similar magnetic sorbent was employed in a one-step extraction and clean-up procedure for the determination of tetracyclines from egg and chicken tissue [79].

3.2.3.4 Multistep Swelling Polymerisation

In this method, spherical particles of latex are synthesised and subsequently swollen in an oil-in-water emulsion of the initiator. The particles absorb the internal phase of the emulsion and are then transferred to another emulsion containing the monomers and the template in the oil phase. Polymerisation is initiated once the monomers and the template have been uploaded by the latex particles, and renders monodisperse particles in the 0.5−50 µm range, with good control of the final size [8, 18, 80]. This strategy was utilised to synthesise MIP of Sudan I for SPE from chilli sauce [81].

3.2.3.5 Core-shell Polymerisation

The synthesis techniques of core/shell nanoparticles can be classified into two types depending on the source of the core particles [82, 83]: (i) the core particles are synthesised and then incorporated into the system after proper surface modification, and (ii) the core particles are synthesised in situ and coated with the shell material. In the latter case, some impurities from the reaction media may be trapped between the core and the shell layer. Shell thickness determines the efficiency of template removal and
the selectivity of the binding; the optimum being at around 10 nm. If the thickness is thinner, selectivity declines considerably. Thicker shells make extraction of the template difficult and do not lead to selective binding [82].

3.2.3.6 Sol-gel Polymerisation

Sol-gel polymerisation enables the preparation of water-compatible MIP, minimising the risk of blockage and deformation of the imprinted cavities due to changes in the degree of swelling [84]. The condensation of organically modified silanes in aqueous media, under mild thermal conditions, leads to hybrid silica-gel or aerogel materials [85]. Firstly, the formation of the organic-inorganic matrix and the imprint process takes place simultaneously through the self-assembly of the alkoxy-silane precursors and the template via hydrolysis/condensation. Secondly, restructuring of the solid matrix occurs during the aging stage. Sol-gel derived materials have a porous structure and can be engineered to have an extremely high surface area. High temperatures can be used to assist the removal process.

3.2.3.7 Grafting Polymerisation

This technique involves the covalent attachment of polymers to particles [86] or membranes [87] used as substrates. During grafting to, reactive preformed polymers are chemically linked to the substrate. In the grafting from stage, the initiator is immobilised on the surface of the substrate and, therefore, growth of the polymer chains occurs from the support surface. An azo-type (AIBN) initiator has been immobilised on polystyrene beads to form MIP for pyrimethanil to selectively extract and preconcentrate the fungicide from wine samples [88]. More advantageous is the use of atom transfer radical polymerisation (ATRP) initiators that generate two radicals via fragmentation; one capable of initiating polymerisation and another that enables recombination between radicals to complete the polymerisation [89]. ATRP initiators prevent polymerisation in the bulk of the solution, providing strict control over the polymer thickness. This strategy has been used to form MIP layers with an average thickness of about 15–20 nm, which could be modulated up to 30 nm by increasing the concentration of monomers [90].

3.2.3.8 In situ Polymerisation

Molecularly imprinted monolithic (MIM) porous polymers can be prepared in situ in a variety of physical forms (e.g., discs and fibres) [91]. The MIM are obtained via a simple, one-step, free radical polymerisation ‘moulding’ process directly within a
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chromatographic column, avoiding the tedious procedures of grinding, sieving and column packing [92]. The polymerisation mixture is degassed and poured into a stainless steel column. After polymerisation, the template and the porogenic solvents are removed by exhaustive washing, usually with MeOH-acetic acid. The large pores present in the MIM enable solvents to elute with low resistance. Convection becomes the dominant mass transportation mechanism, which is much more rapid than diffusion in conventional stationary phases. MIM-based SPE/SPME has been shown to be useful in biological, pharmaceutical, food and environmental analysis, for example, in the in-tube SPME of 8-hydroxy-2′-deoxyguanosine from urine [93], or the SPE of cyromazine and melamine from milk [94] or quinolones from pork samples [95].

3.3 General Recipes

In the last decade, MIP have been used in a variety of extraction techniques such as SPE, MEPS, MSPD, membrane extraction, SPME and SBSE [2] (Figure 3.1). Examples of the composition and synthesis approaches for obtaining MIP useful for these techniques are discussed in the following sections.

3.3.1 Molecularly Imprinted Solid-phase Extraction

MIP are commonly used in the off-line mode of SPE due to the simple instrumentation required and the reduced cost, compared with the on-line process. Nevertheless, the on-line mode has the advantage of working in a closed loop, which reduces pollution, sample contamination and reagent volumes, and improves reproducibility [45].

Bulk polymerisation has been progressively abandoned in favour of particle-based MISPE, although the latter has the disadvantage that a residual amount of stabilisers may remain in the polymer particles even after extensive washing, making rebinding of the template difficult [52]. Recently, new strategies in the synthesis of MIP have been developed [45] and several applications to SPE are summarised in Table 3.1.

3.3.1.1 Materials Based on Magnetic Nanoparticle Composites

Magnetic imprinted particles can provide controllable rebinding of the target molecules from a liquid medium and easy removal of the loaded particles by means of a magnet, avoiding centrifugation and filtration [113, 114]. In addition to conventional heating and UV light methods for polymerisation initiation, microwave heating can be used to rapidly synthesise imprinted layers on Fe₃O₄ nanoparticles [115].