4. CONCLUSIONS

CONCLUSIONS

Les conclusions derivades dels estudis realitzats en la present Tesi Doctoral són les següents:

1. Ha estat possible sintetitzar polímers amb empremta molecular (MIPs) per a una gran varietat de compostos, els quals es diferencien entre ells en aspectes com la mida, la polaritat o el nombre i tipus de grups funcionals.

2. La síntesi de MIPs mitjançant polimerització en solució, tot i haver de triturar i tamissar el polímer abans de ser emprat, ha demostrat ser una tècnica ràpida, relativament senzilla i efectiva per a obtenir MIPs.

3. En la síntesi de MIPs per a un mateix *template* s'ha comprovat com, la via de polimerització seguida o el porogen emprat, influeixen en les característiques finals del MIP.

4. Tot i que és recomanable dur a terme la síntesi de MIPs en solvents orgànics apolars i apròtics, s'ha demostrat com, la síntesi en una mescla d'aigua i un solvent orgànic com a porogen, també pot donar lloc a un polímer amb bones empremtes moleculars i per tant bona selectivitat.

5. L'avaluació cromatogràfica dels MIPs ha estat de gran utilitat per a obtenir una idea sobre les característiques d'empremta molecular de cada MIP i també per conèixer la possible reactivitat creuada que presenta per a altres compostos d'estructura semblant a la del *template*.

6. La reactivitat creuada dels MIPs ha estat aprofitada per a l'extracció selectiva de compostos d'una mateixa família tant en mostres d'aigua de riu com biològiques.

7. S'ha demostrat com els MIPs poden ser emprats tant en sistemes d'extracció en fase sòlida fora de línia com acoblats en línia a la cromatografia de líquids, sempre i quan el solvent d'elució a emprar

sigui compatible amb el sistema cromatogràfic i adient per portar a terme la separació dels compostos.

8. L'ús dels MIPs com a sorbents en sistemes d'extracció en fase sòlida pot ser aplicat a l'extracció selectiva de compostos en matrius tan diverses com la mediambiental i la biològica.

9. Tot i que els MIPs generalment es caracteritzen per tenir baixa capacitat, s'ha demostrat que és possible extreure compostos de naturalesa polar de grans volums d'aigua de riu amb bones recuperacions pels analits extrets.

10. L'etapa de neteja amb un solvent orgànic ha estat clau en molts casos per a assolir una extracció selectiva de l'analit/s d'interès, principalment quan es treballa amb mostres aquoses.

11. L'etapa de neteja junt amb la selectivitat del MIP han permès obtenir cromatogrames molt nets on la matèria orgànica no interfereix en la quantificació de l'analit/s d'interès, la qual cosa és de gran utilitat quan es treballa amb mostres d'elevada complexitat com les de teixit animal.

12. En el cas del MIP preparat amb naproxen com a molècula *template*, l'etapa de neteja permet eliminar gran part de la matèria orgànica present a les mostres d'orina, de manera que ha estat possible variar el gradient de separació per tal que el compost d'interès elueixi en el menor temps possible i així aconseguir temps d'anàlisi més curts.

13. En el cas del MIP empremtat amb ENRO i en el del MIP empremtat amb CIPRO, el procés de MISPE s'ha combinat amb una altra etapa d'extracció prèvia emprant un sorbent comercial d'elevada retenció. Aquest procés d'extracció en dues etapes ha permès realitzar un canvi de solvent, de manera que els compostos que inicialment estan en una

mostra aquosa finalment són preconcentrats a través del MIP en un solvent orgànic en el qual les interaccions selectives són màximes.

14. El procés d'SPE en dues etapes desenvolupat en l'estudi del MIP empremtat amb la CIPRO, ha permés realitzar una neteja de la mostra molt eficaç que, combinada amb la selectivitat del MIP, ha donat lloc a extractes molt nets, la qual cosa ha permès que l'extracte obtingut s'hagi pogut injectar directament en un detector de MS fent innecessària la tècnica cromatogràfica i per tant reduir el temps d'anàlisi.

The conclusions drawn from the various studies of the present Thesis can be summarized as follows:

1. Molecularly imprinted polymers can be synthesised for a wide variety of compounds which are different in size, functional group and polar characteristics.

2. Synthesising MIPs by conventional solution polymerisation is a rapid and relatively easy method for obtaining these materials, although the polymer obtained has to be ground and sieved before it is used.

3. It has been confirmed that for a given template molecule, the synthetic protocol followed and the porogen used affect the characteristics of the polymer.

4 Although it has been stated that MIPs should be synthesised in non-polar and non-protic solvents, this Thesis has demonstrated that imprinted polymers with good imprinting effect can be prepared in mixtures of water and an organic solvent.

5. It is very useful to make a chromatographic evaluation of MIPs to have some idea about the imprinting effect of the polymer and to check the selectivity of the MIP for other analytes that are structurally related to the template molecule.

6. The cross-reactivity shown by the MIPs can be exploited to selectively extract mixtures of compounds from the same group in river water and biological samples.

7. MIPs can be applied in both off-line and on-line solid-phase extraction coupled to liquid chromatography when the eluting solvent is compatible with the chromatographic system and is appropriate for the chromatographic separation.

8. Molecularly imprinted polymers can be applied to the selective solid-phase extraction of compounds from complex matrices such as environmental and biological samples.

9. Although the capacity of MIPs is usually low, it has been demonstrated here that compounds can also be extracted from high sample volumes and good recoveries obtained for the analytes extracted.

10. The clean-up step with an organic solvent is very important in some studies to enhance the selectivity of the MIP, mainly when the target analyte/s has to be selectively extracted from aqueous samples.

11. The clean-up step combined with the selectivity of the MIP allow cleaner chromatograms to be obtained, because the matrix compounds do not interfere in the quantification of the target analyte/s. This is an advantage particularly when complex samples, such as tissue samples, are analysed.

12. In the study of the naproxen MIP, the clean-up step nearly removes the organic compounds from urine. Therefore the separation gradient was changed and the time of analysis decreased.

13. In the studies of the ENRO and CIPRO MIPs, the MISPE process was combined with a previous extraction step in which a commercial SPE sorbent was used. This two-step SPE makes it possible to remove the aqueous content of the sample. Consequently, the analytes pass through the MIP in the appropriate organic solvent.

14. The two-step SPE process performed in the study of CIPRO MIP cleans up the sample more efficiently, so the clean extract obtained from the extraction procedure can be directly injected into a MS detector, avoiding the separation step and decreasing the time of analysis.

ANNEXOS

ANNEX I

Abreviatures utilitzades en la present tesi:

1-NS	àcid 1-naftalensulfònic
4-CP	4-clorofenol
4-NP	4-nitrofenol
4-VP	4-vinilpiridina
ABDV	2,2'-azobis-(2,4-dimetilvaleronitril)
ACN	acetonitril
AIBN	2,2'-azobisisobutironitril
BPO	peròxid de benzoil
CEC	electrocromatografia capil·lar
$CHCl_3$	cloroform
CIPRO	ciprofloxacina
DCM	diclorometà
DMSO	dimetilsulfòxid
DPE	elució amb diferencial de pulsos
DVB	divinilbenzè
ECD	detector electroquímic
EGDMA	dimetacrilat d'etilenglicol
ELISA	assaig amb enzim unit a un immunosorbent
ENRO	enrofloxacina
GC	cromatografia de gasos
GCB	carbó negre grafitat
HAc	àcid acètic
HPLC	cromatografia de líquids d'alta resolució
i.d.	diàmetre intern
IF	factor d'empremta molecular
IS	immunosorbent
K'	factor de capacitat
LC	cromatografia de líquids
LLE	extració líquid-líquid

MAA	àcid metacrílic
MAE	extracció assistida amb microones
MDAA	N,N'-metilendiacrilamida
MeOH	metanol
MIIP	polímers amb empremta d'ió metàl·lic
MIP	polímer amb empremta molecular
MISPE	extracció en fase sòlida mitjançant polímers amb empremta molecular
MMLLE	membranes microporosesd'extracció líquid-líquid
MS	espectrometria de masses
NIP	polímer de control
OTC	oxitetraciclina
PAHs	hidrocarburs aromàtics policíclics
PCA	anàlisi per components principals
PE	elució amb pulsos
PETRA	tetraacrilat de pentaeritritol
PGC	carbó porós grafitat
PLS	regressió per mínims quadrats parcials
PS-DVB	poliestirè divinilbenzè
RAM	material d'accés restringit
RI	índex de retenció
RIA	radioimmunoassaig
SBSE	extracció per sorció amb barres magnètiques agitadores
SLM	membrana de líquid suportat
SPE	extracció en fase sòlida
SPME	microextracció en fase sòlida
TRIM	dimetacrilat de 1,3-propilenglicol
UV	radiació ultraviolada

ANNEX II

Els treballs que han sorgit de la present Tesi Doctoral, inclosos als capítols 1 i 2, que s'han publicat o estan pendents de publicació en revistes científiques, són els següents:

- E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, *Molecularly imprinted polymers applied to the solid-phase extraction of compounds from environmental and biological samples,* Trends Anal. Chem., (pendent de publicació) (apartat 1.3).

- E. Caro, N. Masqué, R.M. Marcé, F. Borrull P.A.G. Cormack, D.C. Sherrington, *Non-covalent and semi-covalent molecularly imprinted polymers for selective on-line soli-phase extraction of 4-nitrophenol from water samples,* J. Chromatrogr. A 963 (2002) 169 (apartat 2.1.1).

- E. Caro, N. Masqué, R.M. Marcé, F. Borrull P.A.G. Cormack, D.C. Sherrington, *On-line solid-phase extraction with molecularly imprinted polymers to selectively extract substituted 4-chlorophenols and 4-nitrophenol from water,* J. Chromatrogr. A 995 (2003) 233 (apartat 2.1.2).

- E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, *Molecularly imprinted solid-phase extraction of naphthalene sulfonates from water,* J. Chromatrogr. A 1047 (2004) 175 (apartat 2.2.1).

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- E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, *Synthesis and application of an oxytetracycline imprinted polymer for the solid-phase extraction of tetracycline antibiotics*, Anal. Chim. Acta (pendent de publicació) (apartat 2.4.1).

- E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, *Novel enrofloxacin imprinted polymer applied to the solid-phase extraction of fluorinated quinolones from urine and tissue samples,* J. Chromatogr. A (pendent de publicació) (apartat 2.5.1).

- E. Caro, R.M. Marcé, P.A.G. Cormack, D.C. Sherrington, F. Borrull, *Direct determination of ciprofloxacin by mass spectrometry after two-step solid-phase extraction using a molecularly imprinted polymer,* J. Chromatogr. A (pendent de publicació) (apartat 2.5.2).

UNIVERSITAT ROVIRA I VIRGILI

Departament de Química Analítica i Química Orgànica

SYNTHESIS AND APPLICATION OF NEW MOLECULARLY IMPRINTED POLYMERS TO THE SOLID-PHASE EXTRACTION

Dissertetion presented by
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to recieve the degree
Doctor of the Rovira i Virgili University
European PhD
Tarragona, 2005

UNIVERSITAT ROVIRA I VIRGILI
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El Dr. FRANCESC BORRULL i BALLARÍN, Catedràtic de Química Analítica del Departament de Química Analítica i Química Orgànica de la Facultat de Química de la Universitat Rovira i Virgili, i

La Dra. ROSA MARIA MARCÉ i RECASENS, Professora Titular de Química Analítica del mateix Departament,

CERTIFIQUEM:
Que la present Tesi Doctoral, que porta per títol: "SÍNTESI I APLICACIÓ DE NOUS POLÍMERS AMB EMPREMTA MOLECULAR A L'EXTRACCIÓ EN FASE SÒLIDA", presentada per ESTER CARO RUBIO per optar al grau de Doctora per la Universitat Rovira i Vigili amb menció europea, 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'esmentada doctoranda.

I, per a que consti, expedim aquest certificat a Tarragona, 10 d'abril de 2005.

Dr. Francesc Borrull i Ballarín   Dra. Rosa M. Marcé i Recasens

M’agradaria donar les gràcies a totes les persones que, d’una manera o *altra, han contribuït a la realització d'aquesta tesi, i especialment:*

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ÍNDEX

OBJECTIU / *OBJECTIVE* 1

1. INTRODUCCIÓ 5

1.1 Extracció en fase sòlida 9

1.2 Polímers amb empremta molecular 17

1.2.1 Síntesi de polímers amb empremta molecular 21

1.2.2 Síntesi de polímers amb empremta molecular mitjançant polimerització en solució 30

1.2.3 Procediment d'extracció en fase sòlida mitjançant polímers amb empremta molecular 42

1.2.3.1 Avaluació preliminar 42

1.2.3.2 Procediment d'extracció en fase sòlida 45

1.2.4 Aplicacions de polímers amb empremta molecular com a sorbents a l'extracció en fase sòlida 52

1.2.5 Noves tendències 63

1.3 *Molecularly imprinted polymers applied to the solid-phase extraction of compounds from environmental and biological samples* 69

1.4 Bibliografia 92

2. PART EXPERIMENTAL I RESULTATS 105

2.1 Polímers amb empremta molecular per a l'extracció de compostos fenòlics mitjançant l'acoblament en línia de l'extracció en fase sòlida 109

2.1.1 *Non-covalent and semi-covalent molecularly imprinted polymers for selective on-line solid-phase extraction of 4-nitrophenol from water samples* 117

2.1.2 *On-line solid-phase extraction with molecularly imprinted polymers to selectively extract substituted 4-chlorophenols and 4-nitrophenol from water* 133

2.2 Extracció en fase sòlida de naftalensulfonats en mostres d'aigua de riu mitjançant l'aplicació d'un polímer amb empremta molecular 143

2.2.1 *Molecularly imprinted solid-phase extraction of naphthalene sulfonates from water* 149

2.3 Polímers amb empremta molecular per a l'extracció d'antiinflamatoris en aigua de riu i orina... 165

2.3.1 *Selective enrichment of anti-inflammatory drugs from river water samples by solid-phase extraction with a molecularly imprinted polymer*.. 171

2.3.2 *A new molecularly imprinted polymer for the selective extraction of naproxen from urine samples by solid-phase extraction* 185

2.4 Síntesi d'un polímer amb empremta molecular preparat amb oxytetraciclina com a molècula template i aplicació a l'extracció en fase sòlida de tetraciclines.. 199

2.4.1 *Synthesis and application of an oxytetracycline imprinted polymer to the solid-phase extraction of tetracycline antibiotics* 205

2.5 Polímers amb empremta molecular per a l'extracció selectiva de fluoroquinolones en mostres biològiques...................................... 217

2.5.1 *Novel enrofloxacin imprinted polymer applied to the solid-phase extraction of fluorinated compounds from urine and tissue samples*.. 225

2.5.2 *Enrichment of fluorinated quinolones from urine samples by a novel ciprofloxacin imprinted polymer as solid-phase extraction sorbent* 239

3. *RESULTS & DISCUSSION* 251

4. CONCLUSIONS / *CONCLUSIONS* 261

ANNEXOS 271

I. Abreviacions utilitzades 273

II. Publicacions 275

OBJECTIU

OBJECTIVE

La present Tesi Doctoral té com a objectiu la síntesi de nous polímers amb empremta molecular i la seva aplicació com a sorbents en processos d'extracció en fase sòlida, per tal de millorar la selectivitat en l'extracció de contaminants ambientals o fàrmacs que es troben en mostres ambientals i biològiques.

The aim of the present Thesis is to synthesise new molecularly imprinted polymers and to apply them as sorbents in solid-phase extraction to improve the selectivity of the extraction procedures when environmental pollutants or drugs are extracted from environmental and biological samples.

1. INTRODUCCIÓ

En els últims anys, un dels objectius principals de la Química Analítica ha estat el desenvolupament de noves tècniques d'extracció que permetin extreure selectivament i eficientment els analits que es troben a la matriu de la mostra i a la vegada preconcentrar-los. Per a l'extracció d'analits presents en mostres líquides, als anys 70 es va desenvolupar, com a alternativa a la clàssica extracció líquid-líquid (LLE), l'extracció en fase sòlida (SPE). Posteriorment, es va desenvolupar la microextracció en fase sòlida (SPME), una modificació de la SPE que disminueix el volum de solvent orgànic a emprar, i més darrerament s'ha desenvolupat l'extracció amb barres magnètiques agitadores (SBSE) per tal d'augmentar la quantitat de sorbent respecte la SPME. L'extracció amb membranes en les diferents modalitats, també ha estat una bona alternativa per a l'extracció de mostres líquides.

De totes maneres, l'SPE s'ha consolidat durant les últimes dues dècades com a tècnica d'extracció d'aquest tipus de mostra. Fins aleshores, la tècnica d'extracció més emprada havia estat l'extracció líquid-líquid [1-4], però degut a les limitacions que presentava (grans volums de solvent orgànic, formació d'emulsions, pèrdua dels analits més volàtils entre les diferents etapes d'extracció, etc...), l'SPE l'ha anat substituint progressivament ja que permet solucionar els inconvenients de la LLE i a més a més permet ser automatitzada fàcilment o acoblada al posterior sistema analític, trets que milloren la reproduïbilitat del mètode d'extracció [5].

L'objectiu de l'SPE i en general el de qualsevol tècnica d'extracció és el d'intentar minimitzar la complexitat de la matriu on es troben els analits a extreure, de manera que després d'aquesta etapa s'obtingui un extracte compatible amb el sistema analític a emprar. L'SPE és una tècnica que es basa en l'ús de sorbents per a l'extracció de compostos que es troben en una mostra líquida. La gran varietat de sorbents existents és un altre dels factors que han afavorit la consolidació de l'SPE com a tècnica d'extracció ja que permet que l'SPE pugui ser emprada per a l'extracció d'analits amb característiques molt diverses.

Una de les principals aplicacions que té l'SPE avui dia es troba dins del camp mediambiental, majoritàriament en la determinació de contaminants, l'interès pels quals ha augmentat considerablement en els darrers anys. La complexitat de les mostres fa necessària una tècnica cromatogràfica per a la seva determinació i el fet que els contaminants es trobin a concentracions baixes, fa imprescindible l'ús d'una tècnica de preconcentració prèvia a l'anàlisi cromatogràfica. A més a més, amb l'ús d'aquesta tècnica de preconcentració també és possible simplificar l'efecte de la matriu de la mostra. Així doncs, l'SPE s'ha aplicat a l'anàlisi de mostres d'aigua de riu, aigua de mar, aigua de consum, aigua residual, etc. Una altra de les grans aplicacions de l'SPE és en l'extracció de compostos d'interès biològic que es troben en biofluids com l'orina, la saliva, el plasma, el sèrum, la sang, etc, o bé en teixits com ronyó, fetge, o múscul, que tot i ser aquestes darreres mostres sòlides, el tractament que se'ls fa abans de l'extracció, per tal d'eliminar partícules sòlides i proteïnes, permet obtenir un extracte líquid. Aquests compostos, tot i que no es troben a nivells tan baixos com els contaminants en mostres ambientals, es troben en mostres que presenten una major complexitat i per tant l'SPE permet simplificar la matriu de la mostra abans que els analits siguin injectats en el corresponent sistema cromatogràfic.

Com a variació de la SPE a començaments dels anys 90 es va desenvolupar la SPME [6], que es fonamenta en els mateixos principis que l'SPE. No obstant, en la SPME la retenció dels analits té lloc en una fibra de sílice fosa la qual es troba recoberta d'un sorbent [7-12]. Malgrat ser una tècnica senzilla, ràpida, de baix cost i poder aplicar-se a l'anàlisi d'una gran varietat de mostres (aigua, mostres biològiques, aliments, aire, etc...) degut a la poca quantitat de sorbent que s'utilitza és més difícil assolir límits de detecció baixos [13]. Per aquest motiu, darrerament s'ha desenvolupat l'extracció amb barres magnètiques agitadores (SBSE) [14] que es basa en els mateixos principis d'extracció que la SPME però que proporciona aproximadament una superfície d'extracció 50 vegades superior. Si bé l'automatització de la SBSE per cromatografia de gasos (GC) està perfectament establerta, igual que la SPME, mitjançant desorció tèrmica, en el cas de la cromatografia de líquids (LC) és més complexa.

L'ús de membranes per a l'extracció de compostos en mostres líquides també ha estat una alternativa a la LLE. Dels tipus de membrana existents cal destacar les no poroses ja que són aquestes les que permeten realitzar una concentració de l'analit de la mostra. Les membranes de líquid suportat (SLM) i les membranes microporoses d'extracció líquid-líquid (MMLLE) es troben dins d'aquest grup. En aquest cas la membrana és activa i la seva estructura química determina la selectivitat de la mostra a transferir [5].

Degut a que la present Tesi Doctoral té com a objectiu la síntesi i aplicació de nous materials selectius per a l'extracció de compostos en mostres líquides mitjançant l'SPE, a continuació s'inclou un apartat on es descriuen les característiques dels sorbents comercials més emprats i dels nous sorbents sintetitzats recentment per tal de millorar-ne les propietats. La recerca d'aquests nous materials va dirigida principalment a millorar aspectes com baixes recuperacions en l'extracció d'analits amb elevada polaritat i la manca de selectivitat [15]. Amb aquest propòsit, darrerament s'han desenvolupat polímers amb monòmer hidrofílic i polímers amb elevat grau d'entrecreuament, entre d'altres, que permeten retenir compostos polars i per tant obtenir bones recuperacions, i per una altra banda s'han sintetitzat els immunosorbents (ISs) i els polímers amb empremta molecular (MIPs), que són materials amb elevada selectivitat que permeten extreure a l'analit d'interès de mostres amb matriu complexa com la mediambiental i la biològica.

1.1 EXTRACCIÓ EN FASE SÒLIDA

L'SPE va ser introduïda a principis dels anys setanta i molts dels sorbents que van ser desenvolupats inicialment han estat utilitzats fins al moment. No obstant, les limitacions que presenten alguns d'aquests sorbents han donat lloc a la recerca de nous materials que permetin minimitzar alguns d'aquests inconvenients.

Els sorbents coneguts com a convencionals o clàssics en processos d'SPE són els de sílices modificades amb C_8 (octacil) o C_{18} (octadecil), molt emprades en processos d'extracció en fase invertida, o modificades amb grups polars com CN (cianopropil), NH_2 (aminopropil) o OH (diol) per a ser emprades en fase normal, els sorbents polimèrics com els de poliestirè-divinilbenzè (PS-DVB), els de base carbó i els bescanviadors d'ions.

Els sorbents de base sílice modificada estableixen principalment interaccions hidrofòbiques tipus Van de Waals amb els analits retinguts. En ser doncs de superfície hidrofòbica, aquests sorbents tenen poca afinitat pels compostos polars i per tant l'extracció d'aquests analits dóna lloc a baixes recuperacions. Per una altra banda, aquests sorbents es caracteritzen també perquè l'interval de pH en el qual són estables és molt limitat i per tant el seu camp d'aplicació es veu reduït.

Els sorbents polimèrics convencionals (PS-DVB) es caracteritzen perquè a més a més d'establir interaccions tipus Van der Waals, també poden formar interaccions π-π entre els anells aromàtics que integren la seva estructura i els dels compostos retinguts. En comparació amb els sorbents de base sílice, els polimèrics retenen millor els compostos de naturalesa polar. No obstant, degut a que l'àrea superficial d'aquests materials és baixa (inferior a 500 m^2 g^{-1}), les recuperacions que s'obtenen en l'extracció de compostos de naturalesa polar encara no són suficientment elevades. Per aquest motiu, en els darrers anys s'han desenvolupat nous sorbents polimèrics funcionalitzats i polimèrics altament entrecreuats que permeten millorar el procés d'extracció tal i com es descriu més endavant. Per una altra banda, cal dir que els sorbents polimèrics convencionals permeten ser emprats en tot l'interval de pH.

Pel que fa als sorbents de carbó hi ha el grafitat (GCB) i el porós grafitat (PGC), ambdós de baixa àrea superficial (100 m^2 g^{-1}) amb bona estabilitat química i tèrmica i capacitat d'adsorció elevada. Degut a aquesta gran capacitat d'adsorció, molts analits queden excessivament retinguts fins i tot de forma irreversible, la qual cosa esdevé un inconvenient perquè la seva elució es fa pràcticament impossible

[1,16]. Tot i així, s'ha demostrat que, compostos orgànics de naturalesa àcida s'adsorbeixen millor en aquest tipus de sorbents que en un C_{18} [16].

Els sorbents bescanviadors d'ions poden ser de base sílice o polimèrica amb ions fixos a la seva estructura. Aquests materials es classifiquen com bescanviadors aniònics si l'ió és una amina o bescanviadors catiònics si l'ió es tracta d'un grup sulfònic o carboxílic. Les interaccions que aquest material estableix amb els analits a extreure són de tipus electrostàtic i per tant presenten una millora pel que fa a les recuperacions de compostos de naturalesa polar respecte a altres sorbents descrits prèviament. No obstant, aquests sorbents de vegades poden veure disminuïda la seva capacitat quan són aplicats a l'extracció de compostos en mostres aquoses degut a la presència d'ions inorgànics que aquest tipus de mostres contenen normalment. Cal destacar que quan aquests sorbents són de base polimèrica mostren una gran estabilitat front a condicions extremes de pH.

Com s'ha comentat, la recerca de nous materials que permetin solucionar o minimitzar les mancances dels sorbents convencionals descrits prèviament és un dels aspectes que desperta més interès dins del camp de l'SPE. Així doncs, aquesta recerca s'ha adreçat principalment a l'obtenció de materials que es puguin aplicar a l'extracció quantitativa de compostos que presenten elevada polaritat en mostres aquoses i, per una altra banda, a l'obtenció de materials que puguin ser aplicats a l'extracció selectiva de l'analit d'interès.

Pel que fa a la millora en les recuperacions d'analits polars, darrerament s'han sintetitzat polímers altament entrecreuats que, degut a l'increment d'àrea superficial que presenten, afavoreixen la retenció d'aquests compostos. Aquests materials s'obtenen generalment mitjançant una polimerització en suspensió amb grans quantitats d'agent entrecreuant, que dóna lloc a polímers amb una àrea superficial de fins aproximadament 800 m^2 g^{-1}, la qual és superior a la dels sorbents convencionals. Aquest increment d'àrea superficial està lligat amb el nombre d'interaccions π-π que el polímer pot establir amb l'analit, les quals en aquest cas augmenten i per tant s'afavoreix la retenció dels analits, de manera que s'obtenen valors de volum de ruptura més grans comparats amb els d'altres sorbents amb menys grau d'entrecreuament [17,18]. Un dels materials altament entrecreuat desenvolupat i comercialitzat amb aquestes característiques ha estat l'Amberlite XAD-4 (≥ 750 m^2/g)

No obstant, si el que es pretén obtenir són polímers altament entrecreuats però amb àrees superficials superiors a 800 m^2 g^{-1}, el procés de síntesi ha de ser mitjançant el post-entrecreuament de les cadenes de poliestirè lineal o del poliestirè lleugerament entrecreuat en presència d'un reactiu bifuncional, d'un solvent i d'un catalitzador de Friedel-Craft. Amb aquests reactius la cinètica de la reacció es veu molt afavorida i es poden arribar a entrecreuar la totalitat dels anells fenílics de l'estructura poliestirènica, d'aquesta manera s'obtenen materials coneguts com hiper-entrecreuats ja que presenten un elevat grau d'entrecreuament, una estructura porosa característica, anomenada biporosa per l'existència a la vegada de microporus i macroporus, que proporciona propietats de sorció molt bones i una àrea superficial molt elevada (800-2000 m^2 g^{-1}) [19]. Els primers treballs desenvolupats en aquest camp van ser duts a terme per Davankov i Tsyurupa l'any 1990 tot i que recentment aquests autors també han publicat altres estudis en aquesta matèria [19-22]. Sychov *et. al.* [23] i Fontanals *et al.* [24,25] són alguns dels autors que darrerament també han realitzat estudis en aquest camp, amb la particularitat de que Fontanals *et al.* van partir d'un monòmer hidrofílic, el qual donava característiques polars al polímer resultant i per tant millorava considerablement la recuperació pels compostos més polars [24]. Són molts els materials que avui dia es comercialitzen amb característiques de sorbents altament entrecreuats; tot i que la informació referent al procés de síntesi no es coneix, la caracterització del material permet determinar la seva àrea superficial, i per tant classificar-los com a hiper-entrecreuats. Així doncs, alguns exemples de materials hiper-entrecreuats amb esquelet d'PS-DVB són l'EnviChrom P (800-950 m^2 g^{-1}) (Supelco), el LiChrolut EN (1200 m^2 g^{-1}) (Merck), l'Styrosorb MN-150 (1070 m^2 g^{-1}) (Purolite Int) i el HYShere-SH (>1000 m^2 g^{-1}) (Supelco), entre d'altres.

Tot i els bons resultats obtinguts amb aquests materials, degut a que la retenció en la majoria d'aquestes reïnes es basa en interaccions hidrofòbiques, les recuperacions pels analits més polars són encara baixes. Una solució a aquest problema és la introducció de polaritat en el polímer, de manera que aquest pot establir interaccions polars amb l'analit en qüestió. Per tal de poder aprofitar els avantatges dels materials amb elevada àrea superficial, darrerament s'han sintetitzat copolímers on es combinen l'addició d'un monòmer que dóna les característiques d'àrea superficial elevada i l'addició d'un altre monòmer que introdueix les característiques polars.

Per preparar aquests sorbents es copolimeritza un monòmer hidrofílic (per exemple, 4-vinilpiridina o N-vinilimidazol) amb un agent entrecreuant (generalment divinilbenzè (DVB)) per tal d'assolir un sorbent que presenti ambdues característiques. En ser utilitzats monòmers més polars que els emprats normalment per a la síntesi d'altres sorbents, és d'esperar que la polaritat del polímer resultant augmenti i per tant que augmenti també la retenció de compostos d'elevada polaritat. Alguns d'aquests sorbents han estat preparats en el nostre grup de recerca en els últims anys [26-29]. Aquests polímers han estat aplicats com a sorbents en processos d'SPE per a l'extracció de contaminants polars (fenols i pesticides) en mostres aquoses i s'ha demostrat que les recuperacions obtingudes amb aquests materials són més altes que les dels materials hidrofòbics. Alguns exemples de sorbents comercials que presenten aquestes característiques són l'Amberlite XAD-7 (450 m^2 g^{-1}) (Rohm & Haas), l'Amberlite XAD-8 (310 m^2 g^{-1}) (Rohm & Haas) i l'Abselut Nexus (575 m^2 g^{-1}) (Varian) on el copolímer es tracta de metracrilat-divinilbenzè (MA-DVB). El Porapak RDX (àrea superficial no disponible) (Waters) i l'Oasis HLB (830 m^2 g^{-1}) (Waters) són altres sorbents hidrofílics que es comercialitzen avui dia on en aquest cas el copolímer emprat és de divinilbenzè i n-vinilpirrolidona (monòmer hidrofílic) [30-32].

Un altre tipus de materials polimèrics altament entrecreuats funcionalitzat són els químicament modificats, l'obtenció dels quals es basa en la modificació química de l'esquelet de les reïnes base de PS-DVB, mitjançant la introducció de grups funcionals polars com l'acetil [33-39], l'hidroximetil [35-39], el benzoil [37-40], el carboxybenzoil [37,41,42], el 2-carboxi-3/4-nitrobenzoil [43], el 2,4-dicarboxibenzoil [43] i els sulfonats [4,37,38,42], etc... Fritz va ser el primer en dur a terme la síntesi d'aquests polímers [35,36,39]. Aquesta modificació del polímer base de PS-DVB té lloc a la superfície del polímer i per tant aquest grup afavorirà el contacte amb l'analit a extreure de manera que es produirà un augment en la recuperació dels analits respecte els seus homòlegs sense modificar [15,33,41,44]. Alguns exemples de sorbents comercials químicament modificats són el Bond Elut PPL (700 m^2 g^{-1}) (Varian), que va ser el primer en ser introduït al mercat i que és de grup funcional desconegut per qüestions de patent, l'Isolute ENV+ (1000-1100 m^2 g^{-1}) (International Sorbent Technology) químicament modificat amb un grup hidroxil, i l'Strata^{TM}X (660-800 m^2g^{-1}) (Phenomenex) de grup funcional no conegut. De totes maneres, per l'extracció de compostos polars s'han obtingut millors recuperacions amb els polímers que contenen un monòmer polar que amb els químicament modificats [45].

Com s'ha comentat, una altra de les fites en la recerca de nous sorbents és la de solucionar la manca de selectivitat que presenten els sorbents convencionals. Entre aquests nous materials aplicats darrerament com a sorbents en processos d'extracció s'inclouen, per una banda els sorbents amb base sílice amb β-ciclodextrines en la seva estructura i els materials d'accés restringit (RAM), i per una altra banda els immunosorbents (ISs) i els MIPs.

Les β-ciclodextrines, que normalment es troben enllaçades a un esquelet de sílice, són oligosacàrids cíclics amb set unitats de glucosa. La cavitat hidrofòbica que es forma presenta una gran capacitat per a establir selectivament complexos d'inclusió amb certes molècules. Així doncs, la selectivitat d'aquest material no ve predefinida per a un analit en concret sinó que el compost que queda retingut acostuma a ser el que presenta una compatibilitat més gran en mida i impediment estèric amb la cavitat de la β-ciclodextrina [46].

Els RAM són materials considerats com selectius degut a que són capaços d'evitar l'accés de la matriu de la mostra a les zones on té lloc la retenció dels compostos. Aquesta restricció pot tenir lloc o bé mitjançant una barrera de difusió física com són els porus de diàmetre concret o bé mitjançant una barrera de difusió química com per exemple una xarxa polimèrica a la superfície de les partícules que es troben en materials semipermeables [15,42,47]. Quan aquests materials s'empren com a rebliment en columnes analítiques, la neteja de la mostra i la separació dels compostos té lloc simultàniament, mentre que quan s'utilitzen com a sorbents d'extracció, aquestes dues etapes es donen seqüencialment. Fins fa uns anys, els RAM només s'empraven per a l'extracció de compostos en mostres biològiques però degut als bons resultats obtinguts, darrerament aquests materials també s'han emprat en l'àmbit mediambiental com, per exemple, per extreure herbicides de mostres aquoses amb elevat contingut d'àcids húmics [47,48]. Alguns dels RAM que es troben disponibles comercialment són el ChromSpher 5 Biomatrix (Chrompack), ISRP GFFII (Regis Technologies), LiChrophere ADS (Merck KGaA).

Quan l'extracció té com a objectiu l'aïllament selectiu d'un compost que es troba entre una mescla de compostos els RAM no ofereixen suficient selectivitat. Així doncs, per tal d'aconseguir tant una neteja selectiva en què només s'eliminin les interferències de la matriu i una extracció selectiva d'un compost, s'han emprat darrerament els ISs (coneguts també com sorbents d'immunoafinitat) i els MIPs [48-51]. En aquests casos, la selectivitat dels processos d'extracció millora considerablement degut a que aquests materials estableixen interaccions selectives amb l'analit d'interès.

En el cas dels ISs, les interaccions selectives es basen en un enllaç reversible tipus antigen-anticòs que s'estableix entre l'anticòs suportat sobre una fase estacionària (sorbent) i l'analit a extreure [16,52]. Els ISs han estat aplicats principalment com a sorbents per a l'extracció d'analits en mostres biològiques i alimentàries, sent més recent la seva aplicació a l'extracció de compostos en mostres mediambientals, degut principalment a la dificultat que hi ha en l'obtenció de ISs selectius per a molècules petites com són les que es troben en aquest tipus de mostra [53-55]. L'extracció emprant aquest tipus de sorbent és tan selectiva que en molts casos permet realitzar en un sol pas l'extracció, la preconcentració i neteja de la mostra [42,56,57].

Tot i la selectivitat que s'obté quan s'empren aquests sorbents en processos d'SPE, sovint els ISs presenten reactivitat creuada. En aquest cas, el sorbent no reconeix a un únic compost sinó que pot extreure diversos compostos de la mateixa família degut a la similaritat estructural que hi ha entre ells [42,58].

Aquesta característica pot ser aprofitada tant en mostres biològiques per tal d'extreure, per exemple, un grup de substàncies farmacològiques i els seus metabòlits principals, com en mostres ambientals, on sovint resulta interessant l'extracció de famílies de compostos, com per exemple herbicides tipus fenilurea o hidrocarburs aromàtics policíclics. No obstant, aquests materials presenten inconvenients pel que fa a la seva síntesi ja que és llarga i costosa, hi ha dificultats per obtenir anticossos selectius per a molècules petites i, a més a més, l'estabilitat d'aquests materials en condicions de pH i temperatura extremes no és molt bona [52]. Tots aquests factors han contribuït a la necessitat de desenvolupar altres materials selectius com alternativa als ISs i que es coneixen com polímers amb empremta molecular [59].

Els polímers amb empremta molecular són materials altament entrecreuats fets a mida per a un analit en concret (molècula objectiu). La selectivitat que presenten aquests polímers és deguda a la presència d'aquesta molècula durant la síntesi del polímer de manera que aquesta queda retinguda a la matriu del polímer resultant. La molècula objectiu es coneix com a molècula *template*, terme àmpliament conegut i emprat en l'àmbit dels MIPs, i que és farà servir d'ara endavant al llarg de la present Tesi Doctoral per referir-nos a la molècula que deixa la seva empremta durant la síntesi del polímer. Els MIPs presenten nombrosos avantatges en comparació amb els ISs ja que són més fàcils d'obtenir, tenen una gran estabilitat tèrmica i química sota condicions experimentals extremes i presenten un camp d'aplicació molt més ampli [52]. Tots aquests avantatges, han donat lloc a que els MIPs siguin emprats cada cop més en nombroses aplicacions, moltes de les quals havien estat desenvolupades utilitzant ISs fins al moment.

Degut a l'interès creixent en assolir extraccions selectives d'analit/s i a la novetat que aquests materials sintètics fets a mida representen, els MIPs han estat l'objectiu de la present Tesi Doctoral. Per aquest motiu, a continuació s'inclou un apartat on es descriuen amb detall les característiques del procés de síntesi, així com les propietats d'aquests materials i la seva aplicació com a sorbents en SPE (MISPE).

1.2 POLÍMERS AMB EMPREMTA MOLECULAR

La tècnica d'empremta molecular és de gran interès actualment dins la comunitat científica tal i com ho demostra el nombre creixent de treballs publicats en els darrers anys. No obstant, tot i que l'interès en la tècnica és molt recent, el concepte en sí mateix presenta una llarga història. L'any 1972 pot considerar-se com el punt de partença d'aquesta tecnologia, quan els doctors Wulff i Klotz per separat van preparar per primera vegada materials orgànics polimèrics amb una selectivitat predeterminada per un lligand. Aquest lligand podia ser considerat com a molècula *template* ja que havia estat present durant la polimerització i per tant el polímer obtingut era un polímer amb empremta molecular.

La relació que existeix entre la molècula *template* i la cavitat del MIP correspon al model conegut com clau-pany que Fischer proposà fa uns 100 anys aproximadament [60]. La tecnologia d'empremta molecular pot ser considerada doncs com una manera de fer panys artificials per a una clau molecular. Amb aquesta tecnologia s'obtenen els MIPs que són una nova classe de materials sintètics fets a mida que presenten elevada selectivitat per un analit en concret. La selectivitat que mostren els MIPs és deguda a la presència de la molècula *template* durant la síntesi del polímer, ja que aquesta interacciona amb el monòmer(s) funcional(s) i forma enllaços específics en presència d'un agent entrecreuant (Figura 1.1). Un cop obtingut el polímer, l'analit emprat durant la síntesi s'extreu de la matriu del MIP i com a conseqüència s'obtenen cavitats o empremtes (*molecular recognition sites*) complementàries en mida i funcionalitat a la molècula *template*. Quan el MIP és utilitzat en qualsevol de les possibles aplicacions, és capaç de reconèixer i retenir selectivament a l'analit emprat durant la síntesi.

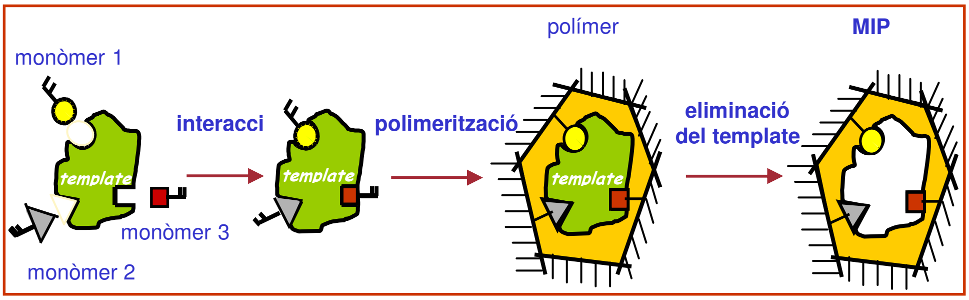


Figura 1.1 Esquema del procés d'empremta molecular.

Les característiques que presenten aquests polímers han permès que es puguin utilitzar per a una gran varietat d'aplicacions, algunes de les quals es troben dins del camp de la Química Analítica i d'altres que es troben una mica més allunyades ja que també poden han estat emprats com a catalitzadors [61], microreactors [62] i dosificadors [63]. Dins de la Química Analítica els MIPs han estat aplicats com a fases estacionàries en HPLC, electrocromatografia (CEC) [64], en processos de SPME i d'SPE [65] i com a receptors en sensors [66].

L'objectiu d'aplicar els MIPs com a sorbents en columnes cromatogràfiques ha estat principalment a la separació de mescles racèmiques de compostos com fàrmacs, amino àcids i sucres. Aquesta separació és possible perquè la síntesi es porta a terme emprant només un dels dos enantiòmers com a molècula *template*. Alguns dels treballs publicats en els darrers anys demostren que és possible preparar MIPs capaços de separar no només enantiòmers sinó també diasteroisòmers [67] i alguns d'ells demostren també que l'eficiència i resolució que presenta el MIP per a un compost és comparable amb el d'algunes fases estacionàries quirals comercials que existeixen avui dia [68]. Aquesta aplicació és de gran interès dins de la indústria farmacèutica degut a la necessitat de purificar els seus productes de substàncies quirals. Una altra de les aplicacions dels MIPs com a fases estacionàries en columnes cromatogràfiques és la separació entre compostos orto- i para- on per exemple un MIP empremtat amb o-aminofenil tetraacetil β-D-galactosidasa com a *template* era utilitzat per analitzar una mescla de para- i orto-aminofenil tetraacetil β-D-galactosidasa. Com era d'esperar, l'analit orto- eluia després del compost para- [69]. Les columnes cromatogràfiques amb MIPs també han estat aplicades per a la separació selectiva d'altres tipus de compostos com poden ser estrògens [70], compostos fenòlics (4-nitrofenol) [71] i aminopiridines (2-aminopiridina i 4-aminopiridina) [72].

Els MIPs també han estat utilitzats com a sorbents en electrocromatografia capil·lar (CEC) [73,74]. En aquesta tècnica, la separació té lloc mitjançant l'aplicació d'un camp elèctric a una columna capil·lar que conté com a fase estacionària un MIP. En aquest cas, per tal de facilitar el procés d'empaquetament de la columna, el MIP es prepara *in situ,* és a dir, dins del mateix capil·lar (procés que es descriu a l'apartat 1.2.1 d'aquest capítol). La CEC també ha estat utilitzada principalment per a la separació d'enantiòmers [75-77]. Tot i que aquesta tècnica no ha estat gaire aplicada fins al moment, la possibilitat que ofereix en la separació d'enantiòmers en petits volums de mostra, fa que sigui una tècnica amb molt de futur.

L'aplicació dels MIPs en processos de microextracció en fase sòlida ha estat desenvolupada recentment per tal de millorar la selectivitat de les fibres en els processos d'extracció. La manera de combinar la SPME amb la tècnica d'empremta molecular és mitjançant el que es coneix com a *in-tube* SPME, on un capil·lar de sílice fosa s'omple amb les partícules del MIP. A continuació, la mostra es introduïda i expulsada del capil·lar diverses vegades de manera que els analits quedin retinguts en el MIP del capil·lar. La desorció dels analits té lloc posteriorment amb la fase mòbil del sistema. El primer estudi realitzat dins d'aquest àmbit va ser desenvolupat per Mullet *et al.* [78], on un MIP empremtat amb propranolol es va immobilitzar a l'interior del capil·lar i es va utilitzar per a l'extracció selectiva d'aquest compost i d'altres compostos de la mateixa família semblants estructuralment. No obstant, Koster *et al.* [79] van ser els primers en preparar un recobriment que consistia en un MIP per a una fibra de SPME. La fibra de base sílice es va silanitzar en una solució de cetona que contenia un 10% de 3-(trimetoxisilil)propil metacrilat i a continuació es va dur a terme la síntesi del MIP a la superfície externa de la fibra. El *template* emprat va ser el clenbuterol i el procés de SPME es va dur a terme per a l'extracció de brombuterol en mostres d'orina humana.

Una altra de les aplicacions dels MIPs dins del camp de la Química Analítica ha estat com elements de reconeixement en sensors. En aquest cas, un senyal químic o físic es genera un cop que l'analit d'interès s'enllaça amb el receptor, MIP, el qual normalment es troba recobrint un transductor. La resposta obtinguda es tradueix en un senyal quantificable que pot variar des de la luminiscència, la colorimetria o la fluorescència, la conductimetria, etc. Els *templates* utilitzats per a la síntesi dels MIPs que s'han aplicat com a sensors són molt variats ja que van des de compostos naturals com la Vitamina K fins a herbicides com l'àcid 2,4-diclorofenoxiacètic [80].

Els MIPs han estat utilitzats també com a substituts dels anticossos en processos de radioimmunoassaig (RIA) on els MIPs han demostrat que poden establir una forta interacció amb l'analit d'interès i a més a més també poden presentar reactivitat creuada, característica pròpia dels ISs. La majoria d'estudis desenvolupats en aquest camp han estat aplicats a l'extracció de compostos farmacològics en mostres biològiques [80].

De totes les aplicacions possibles l'ús dels MIPs com a sorbents selectius en processos d'extracció en fase sòlida (MISPE) és el que més interès ha generat els darrers anys. A continuació s'inclou un apartat on es descriuen els processos de síntesi i obtenció d'aquests materials, la seva aplicació a l'extracció selectiva de compostos en mostres ambientals i biològiques, així com diverses propostes de futur relacionades amb aquests sorbents.

1.2.1 Síntesi de Polímers amb Empremta Molecular

El procés d'empremta molecular pot tenir lloc de diverses maneres en funció de la interacció que es dóna entre el *template* i els monòmers funcionals. Així doncs es pot dir que són tres els protocols que es poden seguir per preparar MIPs:

A) Sistema Covalent (P*re-Organized Approach*):

Com es pot veure a la Figura 1.2, aquest sistema es basa en la formació d'un enllaç fort (covalent) i reversible entre el monòmer funcional i la molècula *template* abans d'iniciar el procés de síntesi formant el que es coneix com a complex *template*-monòmer. Per tant, el *template* ha de ser modificat químicament amb el monòmer funcional abans de començar la polimerització. Un cop obtingut el MIP, el *template* s'elimina de la matriu del polímer per hidròlisi de l'enllaç covalent, generalment mitjançant una extracció Soxhlet.

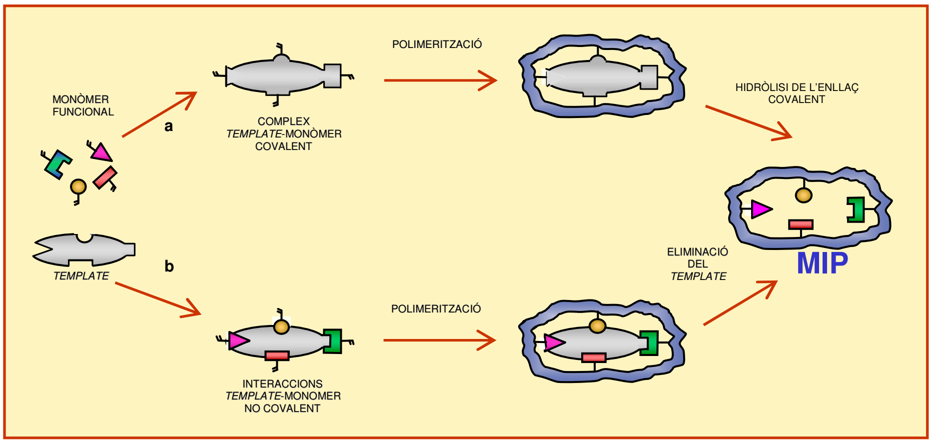


Figura 1.2 Vies de síntesi covalent (a) i no covalent (b) per a l'obtenció dels MIPs.

B) Sistema no Covalent (*Self-Assembly Approach*):

En aquest cas, les interaccions intermoleculars que es donen entre *template* i monòmer funcional són no covalents, com poden ser enllaços d'hidrogen, interaccions iòniques, π-π i hidrofòbiques i per tant són més febles que les del sistema anterior. En aquest cas, la formació del complex *template*-monòmer funcional té lloc durant el procés de polimerització (Figura 1.2), de manera que l'elecció del monòmer funcional és molt important per poder generar cavitats d'enllaç amb elevada afinitat. Un cop obtingut el MIP, el *template* es pot eliminar rentant successives vegades el polímer amb el solvent adequat.

C) Sistema Semicovalent:

En aquest procediment es combinen elements característics del sistema covalent i del no covalent. En aquest cas, el *template* es troba enllaçat covalentment al monòmer funcional formant el complex *template*-monòmer abans de començar la polimerització com en el cas del sistema covalent, però un cop obtingut el MIP el *template* es reenllaça al polímer mitjançant interaccions no covalents com en el sistema no covalent.

En general, els MIPs sintetitzats amb el sistema covalent i semi-covalent donen lloc a cavitats d'enllaç molt més homogènies i millor definides, ja que el complex *template*-monòmer funcional és més estable durant el procés d'empremta molecular que el no covalent. No obstant això, l'aplicabilitat dels sistemes covalent i semi-covalent és més limitada a causa de la dificultat per trobar molècules *template* que puguin ser modificades per formar aquest complex.

El sistema no covalent és molt més flexible que el covalent ja que poden ser emprats com a *template* un major nombre de compostos, i a més a més, el procés de síntesi és més senzill ja que la formació del polímer té lloc barrejant simplement el *template* amb el monòmer funcional sense necessitat de derivatització química. No obstant, els polímers obtinguts mitjançant el sistema no covalent, presenten heterogeneïtat en les cavitats d'enllaç degut a la multitud d'espècies que es formen entre el *template* i el monòmer funcional durant els primers passos de la polimerització. Degut a que l'enllaç no covalent no és tan fort es requereix d'un petit excés de monòmer funcional per tal d'assegurar la formació del complex *template*-monòmer i mantenir-lo estable durant la polimerització. Com a conseqüència, una part dels monòmers funcionals s'enllacen a la matriu polimèrica a l'atzar donant lloc a les cavitats heterogènies que es comentaven abans.

En qualsevol dels tres sistemes, abans de que el MIP pugui ser emprat, cal eliminar de la seva estructura la molècula *template*. No obstant, en alguns casos, la interacció que es dóna durant la síntesi del polímer entre la molècula *template* i el monòmer funcional és tan forta, que un cop obtingut el MIP, és difícil eliminar la molècula *template* quantitativament fins i tot després de successius rentats [81-85]. Aquest fet, anomenat sagnat (*bleeding*) esdevé un problema quan el polímer s'ha d'emprar per la quantificació de compostos a nivell traça ja que en aquesta situació, no només es quantifica l'analit retingut que prové de la mostra sinó que també es quantifiquen restes de la molècula *template*.

Per tal de solucionar aquest inconvenient, alguns autors han sintetitzat MIPs emprant com a molècula *template* un anàleg de l'analit d'interès (*template analogue approach*) [86-91]. Però aquesta solució només és aplicable quan l'ús del MIP és previ a una tècnica de separació (MISPE-HPLC, per exemple), ja que d'aquesta manera si té lloc el sagnat del MIP, el senyal de la molècula *template* (anàleg) no interfereix en la quantificació de l'analit d'interès.

Els primers autors en preparar un MIP emprant un anàleg a l'analit d'interès com a *template* van ser Andersson et al. [84]. En aquest cas, es va preparar un MIP selectiu per la sameridina emprant com a *template* sameridina modificada amb un grup metil addicional. Hi ha altres autors que també han fet ús d'aquesta alternativa [86-91], no obstant, aquesta solució no sempre és viable, perquè sovint no és possible trobar l'anàleg adient o bé la tècnica en la qual s'utilitza el MIP no és de separació. Per aquesta raó també s'han proposat mètodes alternatius que permeten solucionar el problema del sagnat dels MIPs [92]. L'objectiu d'aquests nous mètodes és el de reduir el sagnat del MIP sense haver de recórrer a la necessitat de treballar amb un anàleg de l'analit d'interès com a molècula *template*. Així doncs, Ellwanger *et al.* [92] proposen mètodes de rentat alternatius a l'extracció Soxhlet, com per exemple, l'anelació tèrmica, l'extracció assistida amb microones (MAE), o bé l'extracció amb fluids supercrítics. En aquest treball [92] es fa l'estudi de cadascuna de les tècniques sobre un MIP empremtat amb clenbuterol i comproven com de totes sembla que la més eficient és la MAE, ja que amb aquesta es van obtenir els valors de sagnat més baixos. Per una altra banda, l'extracció amb fluids supercrítics presenta limitacions com són l'elevat cost i la instrumentació requerida. L'extracció Soxhlet va demostrar no ser tan efectiva com la MAE, en reduir el sagnat del MIP només en un 30%.

Un altre aspecte que cal tenir en compte és que de vegades, el MIP no és selectiu només per la molècula *template*, sinó que és capaç de reconèixer selectivament a un grup de compostos semblants estructuralment al compost emprat com a *template*. Aquesta característica coneguda com reactivitat creuada també la presenten els ISs. Tot i que en un principi podria ser un desavantatge, resulta de gran utilitat sobre tot quan el MIP s'utilitza en el camp del mediambient ja que normalment en aquest tipus de mostres s'acostuma a trobar mescles de compostos d'una mateixa família. Hi ha diversos treballs publicats que fan referència a la reactivitat creuada dels MIPs [93-97]. Alguns d'ells, com per exemple els de Ferrer et al. [93] i Matsui et. al. [94], descriuen l'extracció selectiva d'un grup de triazines que es troben en mostres d'aigua. Tot i que aquesta característica s'aprofita principalment per l'extracció de contaminants que es troben en mostres d'origen ambiental, també hi ha estudis on el MIP presenta reactivitat creuada i s'ha aplicat a l'extracció de compostos en mostres biològiques com bilis, sèrum o plasma per extreure selectivament a famílies de compostos farmacològics com el propranolol i els seus anàlegs [98], agents letals emprats en guerres químiques i els seus productes de degradació (metilfosfonats) [99], o nicotina i els seus productes d'oxidació [100].

Pel que fa al procediment de síntesi, els MIPs poden ser sintetitzats emprant bàsicament les següents tècniques de polimerització [101,102]: solució convencional, precipitació, suspensió, inflament en dues etapes (*two-step swelling*) i emulsió miscel·lar (*emulsion core-shell*). Cadascuna d'aquestes tècniques presenta diferents característiques, tal i com es mostra a la Taula 1.1, i dóna lloc a polímers amb diferents propietats

Taula 1.1 Tècniques de polimerització més emprades en la síntesi de MIPs.

Tècnica de polimerització	Polímer que s'obté	Avantatges	Inconvenients
Solució	Monòlit	-Senzill -Baix cost	-Triturar i tamisar el MIP -Possible trencament de les cavitat d'enllaç en triturar el MIP -Partícules amb mida irregular
Precipitació	Partícules esfèriques regulars	-Senzill -No cal triturar ni tamisar -Cavitats d'enllaç homogènies	-Ús de grans volums de solvent

Suspensió en aigua	Partícules esfèriques	Ràpida	-Presència d'aigua durant la síntesi
Suspensió en fluorocarbons		No presència d'aigua	-Elevat preu dels fluorocarbons -Dificultat en estabilitzar mescla de polimerització
Inflament en dues etapes	Partícules esfèriques monodisperses	Control de la mida de les partícules	-Llarga i laboriosa -Presència d'aigua durant la síntesi
Emulsió miscel·lar	Partícules monodisperses	Morfologia molt estructurada	-Presència d'aigua durant la síntesi

El primer mètode de polimerització emprat per a la síntesi de MIPs va ser el de polimerització en solució [103,104]. Aquest mètode ha estat el més utilitzat fins ara degut a la seva simplicitat i universalitat ja que consisteix bàsicament en barrejar tots els components en un solvent orgànic. Com a resultat, s'obté un polímer en forma de monòlit que s'ha de triturar i tamisar per tal d'obtenir-lo en forma de partícules i poder empaquetar les columnes d'extracció. Aquesta via de síntesi és la que s'ha emprat per a obtenir els MIPs sintetitzats en la present Tesi Doctoral, per aquest motiu a l'apartat 1.2.2 d'aquest capítol es descriu amb més detall quin és el procediment a seguir. Aquest mètode també presenta algunes limitacions com per exemple que la distribució de la mida de partícules és aleatòria i que cal triturar i tamisar el polímer, amb la conseqüent pèrdua de les partícules de mida més petita durant aquestes etapes, i la possibilitat de trencament de les cavitats d'enllaç [101,102,105].

La polimerització per precipitació és un mètode que permet obtenir microesferes amb una mida d'aproximadament 1 µm i cavitats d'enllaç més homogènies que les obtingudes en la polimerització en solució. Aquesta metodologia consisteix en la polimerització de la barreja de reactius (*template*, monòmer, entrecreuant) en presència d'una gran quantitat de solvent (porogen), sistema que resulta molt més diluït que l'anterior sistema en solució [101,105-109]. Aquest mètode també és senzill i presenta com a principal avantatge el fet que no cal triturar ni tamisar el polímer un cop sintetitzat. Tot i la regularitat de les esferes obtingudes, cal remarcar que en ser d'una mida tan petita (1 µm) la seva aplicació com a sorbents en columnes cromatogràfiques no és adient. Per aquest motiu, altres autors com Wang *et. al* [110] en els darrers anys han adreçat les seves investigacions a l'obtenció d'esferes amb una mida superior (3-5 µm) de manera que fossin ideals per a ser emprades com a sorbents en cromatografia de líquids.

Fins a mitjans dels anys 90, la síntesi de MIPs es duia a terme únicament en solvents orgànics com a porògens, i per tant compostos solubles en aigua no eren gaire emprats com a *template*s. Per tal de solventar aquesta limitació, es van desenvolupar mètodes de síntesi utilitzant sistemes aquosos com la polimerització en suspensió i la polimerització per etapes [102,111,112].

La polimerització en suspensió [113-115] és un mètode senzill que consisteix bàsicament en barrejar els monòmers continguts en un solvent orgànic amb un excés d'aigua que conté un agent estabilitzant (alcohol polivinílic o polivinilpirrolidona). Les dues fases es barregen vigorosament per agitació fins a obtenir una suspensió de gotetes de solvent que estan contingudes a la fase aquosa [101]. Degut a que les gotes que es formen durant la polimerització són petites i es barregen vigorosament, totes aquestes gotes se situen molt a prop de les parets del reactor, de manera que quan la reacció té lloc mitjançant radiació ultraviolada (UV), la radiació pot arribar a tota la suspensió malgrat que es tracti d'una suspensió blanca i no transmissiva. La mida final de les partícules ve determinada per la mida de les gotes que es formen durant la polimerització i aquestes a la vegada depenen de la força d'agitació emprada durant la barreja de les fases, de la naturalesa de les fases i de la quantitat d'estabilitzant emprat. Altres factors que també influeixen en el polímer resultant són el disseny i geometria del reactor així com la temperatura. Les partícules esfèriques que s'obtenen poden presentar un diàmetre d'entre 5 i 50 µm.

Però aquest sistema en suspensió no sempre és viable per a obtenir MIPs tot i que el *template* sigui soluble en aigua, ja que l'aigua pot interferir en la formació d'interaccions entre el *template* i el monòmer funcional i per tant l'efecte d'empremta molecular podria veure's reduït, sobretot quan s'estableixen interaccions d'enllaç d'hidrogen [101]. En canvi, si les interaccions que es donen entre el *template*-monòmer funcional són electrostàtiques o hidrofòbiques, però suficientment fortes, aquest problema disminueix. Per tal de solucionar aquest inconvenient, però aprofitar els avantatges que aquest mètode ofereix, s'ha dut a terme la síntesi en medis alternatius com per exemple en fluorocarbons [116,117]. La síntesi en fluorocarbons com a solvents per a obtenir MIPs va ser descrita per primera vegada per Mayes i Mosbach [116]. Aquest solvent és immiscible amb altres compostos orgànics, és químicament inert, per tant no pot interferir en les interaccions del procés no covalent, i no és tòxic. No obstant, els fluorocarbons presenten un elevat cost [108] i a més la mescla de polimerització és difícil d'estabilitzar degut a la densitat d'aquest solvent [118]. Fins al moment, no hi ha molta bibliografia que faci referència al desenvolupament de nous surfactants que permetin solucionar aquests problemes.

La polimerització per inflament en dues etapes (Figura 1.3) aplicada a la síntesi de MIPs es va ser dur a terme per primer cop per Hosoya *et al.* l'any 1994 [119]. Aquest mètode implica diverses etapes fins a obtenir el MIP resultant. Inicialment, a partir d'estirè i aigua s'obtenen unes partícules de làtex mitjançant una polimerització per emulsió amb agitació mecànica. Un cop obtingudes les partícules, aquestes s'addicionen a una mescla que conté un agent estabilitzant (dodecilsulfat de sodi) dissolt en aigua i un solvent activant com pot ser el dibutil ftalat. En aquestes condicions té lloc el primer inflament de les partícules. Després de diverses hores d'inflament, a aquesta mescla se li afegeix el *template*, el porogen, el monòmer funcional i un iniciador radicalari, tots ells dispersats en una solució d'aigua, i un estabilitzador polimèric com és l'alcohol polivinílic. La mescla s'agita durant unes hores i es purga amb un gas inert abans de ser introduïda en un reactor on s'augmenta la temperatura i té lloc la polimerització durant 24 hores.

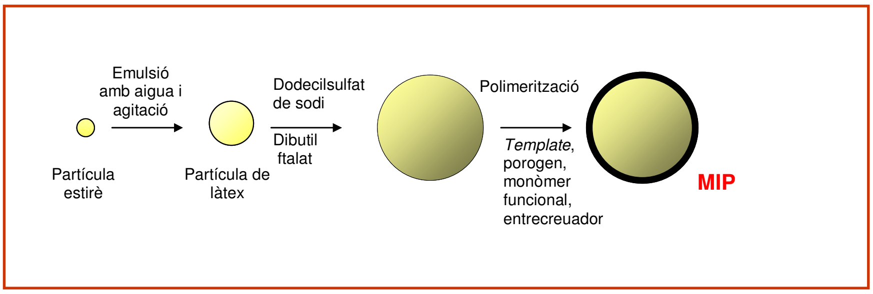


Figura 1.3 Esquema del procés de síntesi per inflament en dues etapes.

Aquest mètode permet un bon control de la mida final (2- 50 µm) i del nombre de partícules que es produeixen. En general es pot dir que aquest mètode és llarg i laboriós [120] però, tot i la presència d'aigua durant la síntesi, també s'obtenen MIPs amb bones característiques d'empremta molecular. Una variació d'aquest mètode consisteix en preparar MIPs mitjançant inflament (*swelling*) i polimerització per suspensió a la vegada, de manera que presenta els avantatges de la polimerització per inflament en dues etapes però és més senzilla i no tan llarga [121].

Les partícules tipus miscel·la (*core-shell*) s'obtenen a partir d'una polimerització per emulsió miscel·lar [122-124]. Aquestes partícules presenten una morfologia molt estructurada que permet la incorporació de qualsevol propietat al nucli de la partícula (*core*) sense interferir en la capa exterior empremtada (*shell*). Les partícules que s'obtenen són monodisperses i es formen en aigua com a medi de polimerització. Aquesta polimerització té lloc en dues etapes, a la primera es forma una partícula de làtex que es pot preparar a partir d'una gran varietat de materials (estirè, divinilbenzè, alquil acrilat...). Aquestes partícules són generalment monodisperses i presenten una mida entre 0.03 i 1 µm de diàmetre. En la segona etapa les partícules obtingudes es barregen amb una mescla de monòmers. La mida, la morfologia i les propietats físico-químiques de les partícules tipus miscel·la del polímer es poden controlar mitjançant la composició i l'estructura dels monòmers i les condicions de reacció.

Altres mètodes de polimerització, tot i que no són tan emprats, són la polimerització per aerosol [125] que com el seu nom indica, la síntesi té lloc en aerosols i no en suspensió i per tant la necessitat d'emprar grans volums d'agent dispersant disminueix i no hi ha problemes d'interferències amb l'agent dispersant, i la polimerització a la superfície de partícules de làtex [126] adreçada específicament a l'obtenció de cavitats d'enllaç amb bona accessibilitat i aplicada principalment a l’extracció d’ions metàl·lics. En aquest procediment de síntesi, les cavitats d’enllaç es formen a la superfície de les partícules de làtex mitjançant la disposició de les cadenes del polímer a la interfase formada per l’aigua i el làtex seguit d’una segona polimerització per tal de fixar aquesta conformació a l’espai. Aquesta metodologia també dóna lloc a l’obtenció de partícules uniformes.

Com a mètodes de síntesi per a obtenir MIPs cal parlar també dels que fan referència a l’obtenció d’aquests materials *in situ.* El procés d’empremta molecular *in situ* pot ser definit com una tècnica per preparar MIPs en el mateix lloc on el polímer ha de ser emprat posteriorment [127]. Aquest mètode per tant no requereix d’un tractament per triturar, tamisar o filtrar el polímer un cop obtingut. L’únic tractament que se li ha de fer a aquest material és rentar-lo per tal d’extreure la molècula *template* de les cavitats. Els dispositius que s’empren per a obtenir MIPs *in situ* són tres principalment: columnes d’acer inoxidable per a que el MIP sigui emprat com a columna cromatogràfica o bé d’SPE [128-131], capil·lars per a ser emprats en sistemes electroforesi capil·lar o electrocromatografia capil·lar [132]; i un vial per a un ús en discontinu o *batch*, per a la determinació de la saturació de les cavitats d’enllaç del polímer [133-135].

A continuació es tracta en detall el procés de síntesi mitjançant la polimerització en solució.

1.2.2 Síntesi de MIPs mitjançant polimerització en solució

El mètode de polimerització en solució permet obtenir aquest tipus de polímers d’una manera relativament ràpida i senzilla. Per tal d’aprofitar aquestes dues característiques es va emprar aquest mètode de polimerització per preparar els MIPs ja que el fet d’obtenir partícules irregulars no suposa una limitació molt important quan els MIPs són emprats com a sorbents en processos d’SPE [120].

Per tal de sintetitzar un MIP en format monòlit són necessaris a més de la molècula *template*, un o diversos monòmers funcionals, un solvent (porogen), un agent entrecreuant i un iniciador. A continuació es descriu amb detall com influeixen cadascun d'aquests reactius en les característiques finals del MIP, el paper que juguen durant la síntesi i quins són els reactius més freqüents de cada tipus.

a) *Template*

La molècula *template* té un paper rellevant durant el procés d'empremta molecular, ja que en funció de les característiques d'aquesta molècula es triarà un monòmer funcional o un altre. Un bon *template* ha de ser estable en les condicions de polimerització seleccionades i tenir grups funcionals que puguin formar enllaços amb el(s) monòmer(s) funcional(s). Són molts i diversos els compostos que fins al moment han estat emprats com a *template*s, alguns d'ells són compostos d'interès biològic com naproxen [136], propranolol [137], nicotina [138,139], teofilina [133,140,141], colesterol [123,142,143] o cafeïna [144] entre d'altres. Alguns herbicides i pesticides com triazines [66,119,135,145-149], l'àcid 2-fenilpropiònic [150], bentazona [151], l'àcid 2,4-diclorofenoxiacètic [152,153] i alguns contaminants ambientals (principalment en mostres aquoses) com compostos fenòlics [154] també han estat emprats com a *template*s en processos de síntesi de MIPs. En altres treballs, macromolècules com proteïnes (sèrum albúmina bovina [155], cytochrom C [156], ribonucleasa [156,160]) han estat compostos utilitzats com a *template;* no obstant, el fet que aquests tipus de molècules presentin nombrosos grups funcionals, limitada estabilitat front temperatura i pH i baixa tolerància als mètodes convencionals de síntesi de MIPs fa que l'ús d'aquests compostos com a *template* estigui limitat i a més a més en ser molècules grans la seva eliminació de l'estructura del MIP no és senzilla.

b) Porogen

El *template* ha de ser soluble en el solvent de polimerització de manera que la reacció es porti a terme amb tots els reactius en una sola fase. El solvent que influeix directament en la formació dels porus en l'estructura d'un polímer, se'l coneix amb el nom de porogen. És molt important l'ús d'un bon porogen en la síntesi de MIPs, ja que l'estructura del polímer resultant i la mida dels porus facilitaran l'accés de l'analit a les cavitats d'enllaç un cop obtingut el polímer. Un porogen ideal és aquell que no interfereix en les interaccions entre el monòmer funcional i el *template* durant la polimerització, ja que podria desestabilitzar el complex que es forma entre ells dos i per tant s'obtindrien polímers amb baix reconeixement molecular [158]. Com s'ha comentat anteriorment, el sistema no covalent és el més emprat en la preparació de MIPs, i per tant les interaccions que es donen solen ser d'enllaç d'hidrogen, hidrofòbiques, π-π o electrostàtiques i; per aquest motiu, porògens apròtics de baixa polaritat i baixa capacitat per formar enllaços d'hidrogen són els més adients [159]. Alguns dels solvents més emprats són els que es mostren a la Taula 1.2.

Taula 1.2 Porògens més emprats i les corresponents constants dielèctriques.

Porogen	**Constant dielèctrica (ε_r)**
Cloroform	5
Diclorometà	9
Toluè	2
Tetrahidrofurà	8
Acetonitril	36
Aigua	80

Per una altra banda el porogen té també una influència directa en l'estructura del MIP que s'obté: àrea superficial i diàmetre del porus. MIPs amb baixa macroporositat esdevenen polímers amb menys reconeixement per la molècula *template* degut a la poca accessibilitat a les cavitats d'enllaç [50].

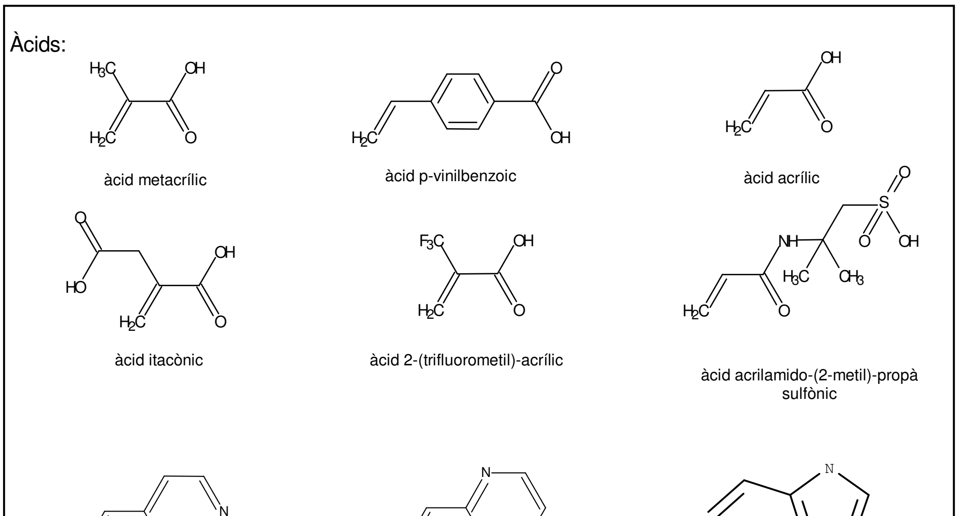
El porogen també juga un paper molt important un cop obtingut el MIP, ja que quan el polímer és emprat en qualsevol de les possibles aplicacions, se suposa que l'analit d'interès es reenllaça millor (hi ha més reconeixement de la molècula *template*) amb el MIP si aquest té lloc en el mateix solvent emprat durant la síntesi (*solvent memory effect*) [91,93,96,160,161] ja que en aquesta situació es reprodueixen les condicions que s'estableixen durant la formació del polímer.

Aquest aspecte suposa un problema principalment quan l'analit es troba en mostres de naturalesa aquosa. La presència d'aigua durant el procés de reconeixement pot disminuir la capacitat del MIP per reconèixer la molècula *template* ja que l'aigua pot trencar les interaccions específiques sobre tot quan aquestes són no covalents. No obstant, en els darrers anys s'han publicat diversos treballs que demostren que també és possible extreure selectivament i amb bones recuperacions analits que es troben en mostres aquoses mitjançant MIPs que han estat preparats en porògens polars [154].

En alguns treballs publicats darrerament, s'ha demostrat que, els MIPs també es poden preparar utilitzant com a porogen una mescla de dos solvents, dels quals normalment un d'ells és aigua i l'altre és un solvent orgànic (per exemple metanol) o bé de dos solvents polars on un d'ells és pròtic (per exemple metanol/acetonitril) [162-165]. La presència d'aigua en aquestes barreges és minoritària però suficient per afavorir en molts casos la solubilitat de la molècula *template* en la mescla de polimerització. Tot i que en un principi caldria esperar que les interaccions específiques no es formessin degut a la presència d'aigua, amb els resultats obtinguts en els treballs citats prèviament, queda de manifest que els MIPs preparats amb aquest porogen donen lloc a polímers amb bona selectivitat. Un altre exemple de MIPs preparats en presència d'aigua són els que s'obtenen mitjançant la polimerització per inflament en dues etapes, els quals han demostrat tenir també una bona selectivitat [166,167].

c) Monòmer funcional

El monòmer funcional és responsable de les interaccions d'enllaç que es donen a les cavitats dels MIPs. La funcionalitat del monòmer ha de ser complementària a la de la molècula *template* per tal de potenciar al màxim l'efecte d'empremta molecular [168]. A la Figura 1.4 es mostren els monòmers funcionals emprats habitualment i classificats segons les seves propietats àcides, bàsiques o neutres.



àcid metacrílic

àcid p-vinilbenzoic

àcid acrílic

àcid itacònic

àcid 2-(trifluorometil)-acrílic

àcid acrilamido-(2-metil)-propà sulfònic

Bàsics:

Neutres:

Figura 1.4 Monòmers funcionals emprats habitualment per la síntesi de MIPs.

Normalment, per a *template*s amb grups funcionals àcids els monòmers funcionals utilitzats són bases de Brönsted (2- o 4-vinilpiridina, per exemple), mentre que per a *template*s amb grups funcionals bàsics són emprats monòmers funcionals amb característiques àcides (àcid metacrílic o àcid trifluorometilacrílic, entre d'altres).

De tots ells, l'àcid metacrílic és el monòmer funcional més utilitzat degut a la seva doble funcionalitat, ja que per una banda el grup carboxílic pot establir enllaços d'hidrogen i per una altra actuar tant donant protons com a acceptant. A més, aquest monòmer funcional també és idoni per establir interaccions iòniques. En alguns treballs s'han emprat mescles de dos monòmers funcionals per potenciar l'efecte d'empremta molecular o bé perquè l'analit té grups funcionals de diverses característiques [151,169-171]. En qualsevol dels casos, és important no afegir monòmer funcional en excés, ja que el sobrant no forma cavitats d'enllaç específiques sinó que dóna lloc a punts de reconeixent no específics.

d) Agent entrecreuant

Per tal d'obtenir cavitats d'enllaç ben definides i mantenir l'agrupació *template*-monòmer funcional durant la polimerització, de manera que els grups funcionals quedin fixats a l'espai de forma complementària al *template*, és necessària la presència d'un agent entrecreuant, el qual normalment es troba en el polímer en una proporció del 80% [173].

L'agent entrecreuant juga també diversos papers durant la síntesi ja que per una banda dóna rigidesa a la matriu polimèrica i per una altra controla la morfologia, és a dir, que les cavitats d'enllaç siguin accessibles. La majoria de polímers presenten una àmplia distribució de la mida dels porus, així doncs, les cavitats d'enllaç associades amb meso- i macroporus (>20 Å) seran més accessibles que aquelles localitzades en zones microporoses (<20 Å) on la difusió és menor [168,172].
La Figura 1.5 inclou els agents entrecreuants més emprats per a la síntesi de MIPs, la majoria dels quals són comercials. La selecció d'un o d'altre dependrà de la seva solubilitat en el medi de polimerització. De tots ells, els més emprats són el dimetacrilat d'etilenglicol (EGDMA), el dimetacrilat d'1,3-propilenglicol (TRIM), el p-divinilbenzè (DVB), la N,N'-metilendiacrilamida (MDAA) i el tetraacrilat de pentaeritritol (PETRA).

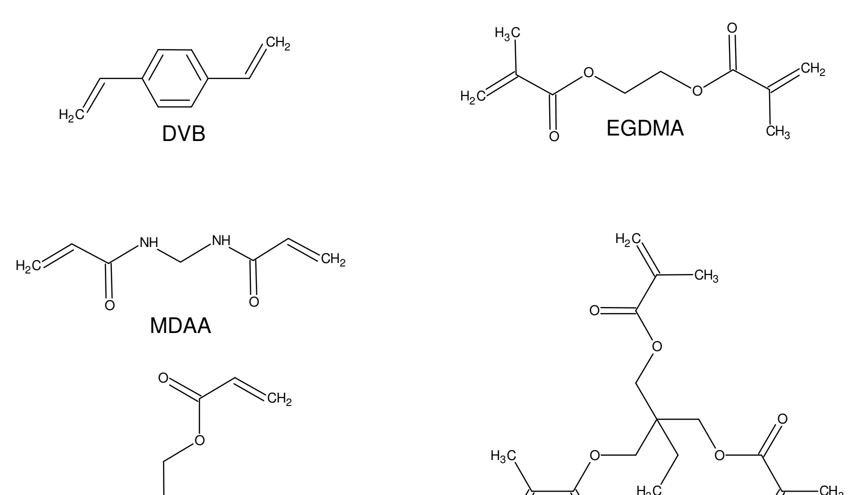


Figura 1.5 Agents entrecreuants més emprats.

L’EGDMA juga un paper molt important en la majoria de sistemes metacrílics (monòmer funcional d’aquestes característiques també) ja que aquest entrecreuant proporciona MIPs amb bona estabilitat mecànica i tèrmica, amb ràpida transferència de massa i bones característiques de reconeixement. Es pot dir que aquest agent entrecreuant dóna lloc a MIPs amb molt bones propietats i és capaç d’interaccionar amb una àmplia varietat de *templates* [158]. El TRIM proporciona polímers amb elevada capacitat de càrrega, mentre que el MDAA i PETRA s’empren en la síntesi de MIPs on el porogen és de característiques més polars.

e) Iniciadors

Pel que fa als iniciadors, poden classificar-se principalment en dos tipus: fotoiniciadors i termoiniciadors. Els més emprats per a la síntesi de MIPs són el 2,2’-azobis-(2,4-dimetilvaleronitril) (ABDV) i l’azobis-isobutironitril (AIBN), les estructures dels quals es mostren a la Figura 1.6. Aquests dos iniciadors descomposen per calor a 40ºC i 60ºC, respectivament, o bé per radiació ultraviolada visible. La polimerització que té lloc és radicalària i es produeix en tres etapes: iniciació, propagació i terminació. Per tal d'evitar un acabament de la polimerització abans d'hora s'ha d'eliminar tota presència d'oxigen a la mescla de polimerització, ja que l'oxigen té molta facilitat per captar els electrons dels radicals formats durant la reacció. És per aquest motiu que tots els reactius (*template*, entrecreuant...) se sotmeten a un tractament previ per tal d'eliminar possibles antioxidants que inhibirien la polimerització. La mescla també es purga amb nitrogen durant 5 minuts en un bany de gel abans d'iniciar la reacció per tal d'eliminar l'oxigen que hi pugui haver dissolt.

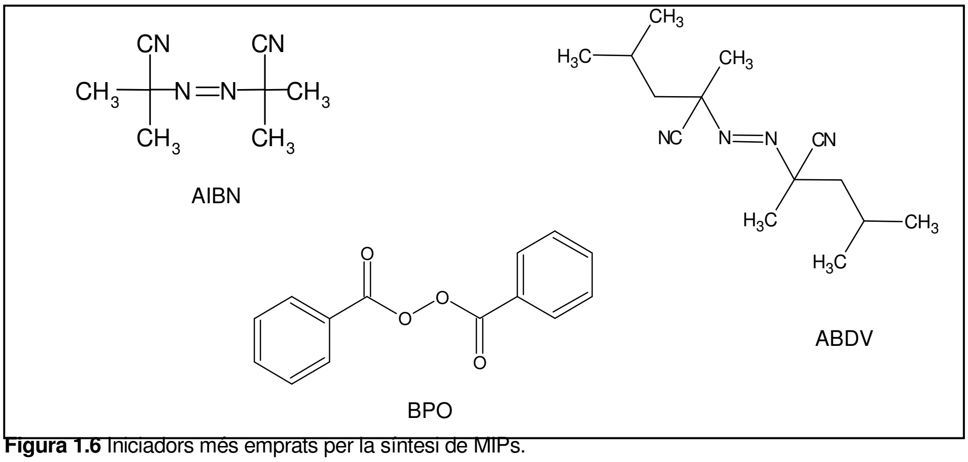


Figura 1.6 Iniciadors més emprats per la síntesi de MIPs.

Per tal de seleccionar l'iniciador cal tenir en compte també les propietats de la molècula *template* emprada, és a dir, si és o no estable tèrmicament o fotoquímicament. Cal avaluar les interaccions que es donaran entre el *template* i el monòmer funcional, ja que si són enllaços d'hidrogen és millor treballar a baixes temperatures durant la polimerització i per tant és millor emprar iniciadors actius fotoquímicament, ja que aquests són efectius fins i tot a temperatures sota zero [168] .

El monòmer funcional, el porogen, l'agent entrecreuant i l'iniciador, són els reactius que intervenen en la síntesi d'un MIP. Per tal d'obtenir un polímer amb empremta molecular en forma de monòlit cal addicionar cada reactiu seguint les etapes que es descriuen a continuació:

- Primera etapa

La molècula *template* es dissol en el porogen juntament amb els diferents monòmers funcionals que també intervenen en la polimerització. Si els monòmers funcionals són líquids, normalment contenen inhibidors que eviten la seva degradació i per tant s'han d'eliminar abans de ser emprats [173]. En aquesta primera etapa, comencen les interaccionar entre els diferents reactius addicionats (Figura 1.7).

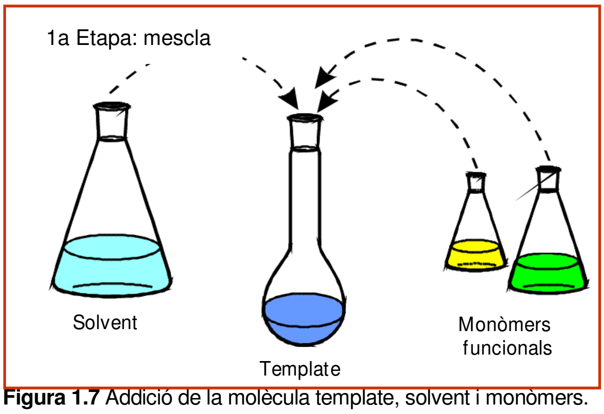
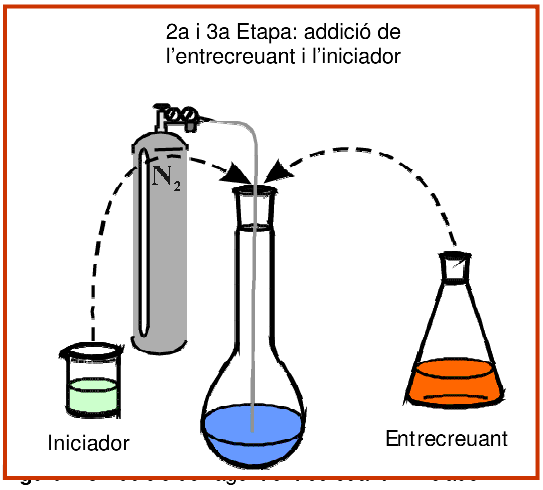


Figura 1.7 Addició de la molècula template, solvent i monòmers.

Els monòmers funcionals són unitats químiques polimeritzables que presenten grups funcionals com carboxil, hidroxil, amino o grups aromàtics. Com s'ha comentat anteriorment, el monòmer funcional seleccionat per a la síntesi del polímer (o conjunt de monòmers) dependrà generalment de les característiques àcides o bàsiques de la molècula *template* [174].

- Segona etapa

En aquesta etapa s'afegeix una gran quantitat d'agent entrecreuant (Figura 1.8) per a que el polímer obtingut sigui altament entrecreuat amb elevada rigidesa i insolubilitat, característiques necessàries per tal d'assegurar que el polímer pugui mantenir l'estructura de la cavitat complementària en mida i funcionalitat a l'espècie que produeix l'empremta.



- Tercera etapa

Addició de l'iniciador. És l'últim reactiu que s'afegeix a la mescla de reacció i normalment sol ser un iniciador per a polimeritzacions radicalàries, com l'AIBN o el BPO. Immediatament després i abans de tancar el tub de polimerització, la mescla es purga durant 5 o 10 minuts amb un gas inert per eliminar del medi l'oxigen present (Figura 1.8).

- Quarta etapa

La polimerització pot iniciar-se o bé amb augment de temperatura tal i com es mostra a la Figura 1.9, o bé amb radiació ultraviolada a baixes temperatures (normalment entre 0 i 5ºC). Tot i que la polimerització a baixes temperatures afavoreix que la formació de complex *template*-monòmer funcional sigui més estable, sovint no és possible portar a terme la polimerització mitjançant radiació ultraviolada degut a la inestabilitat de la molècula template en aquestes condicions [158,175].

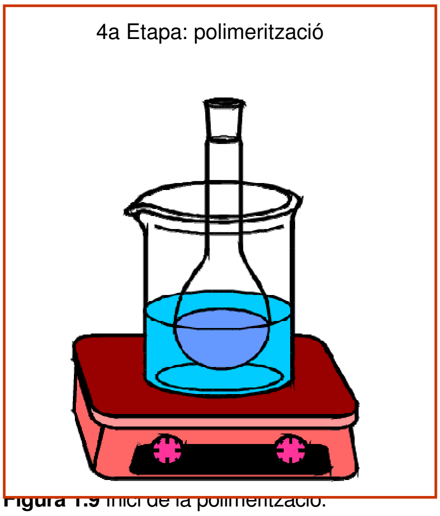


Figura 1.9 Inici de la polimerització.

En moltes ocasions la polimerització té lloc primer mitjançant un procés de radiació ultraviolada durant 24 h a baixes temperatures i posteriorment el polímer obtingut se sotmet a un tractament tèrmic a 60ºC per tal de donar-li les propietats mecàniques adients.

-Cinquena etapa

En aquest tipus de polimerització s'obté un polímer rígid anomenat monòlit. Per extreure'l cal trencar el tub on ha tingut lloc la polimerització tal com es mostra la Figura 1.10. A continuació, per tal d'emprar el MIP com a sorbent en SPE, el polímer es tritura i es tamisa per a obtenir partícules de mida adequada, normalment entre 25-40 µm. Abans d'emprar el MIP per a qualsevol de les aplicacions, cal rentar-lo per tal d'eliminar la molècula *template* de la matriu del polímer i deixar les cavitats del MIP buides per que l'analit d'interès pugui reenllaçar-se selectivament en aplicacions posteriors.

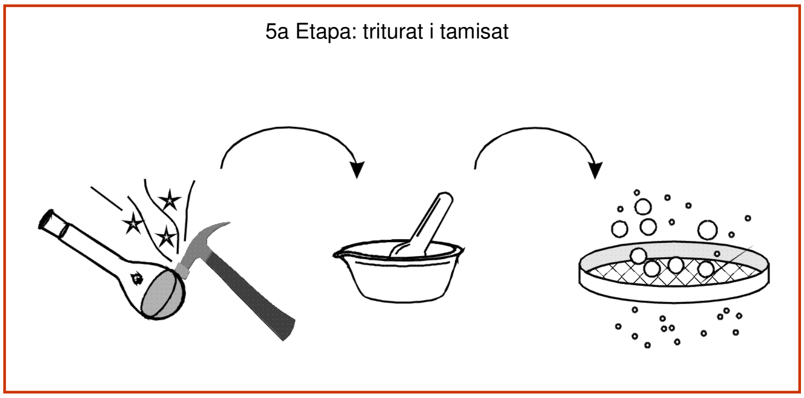


Figura 1.10 Obtenció del monòlit, trituració i tamisat del MIP

Normalment, de forma simultània a la síntesi del MIP, es prepara també el que es coneix com polímer de control (*non-imprinted polymer*, NIP), el qual s'obté emprant els mateixos reactius descrits per preparar el MIP a excepció de la molècula *template*. Com a conseqüència, durant la síntesi del NIP no hi ha formació de les cavitats de reconeixement molecular (empremtes). Aquesta diferència entre ambdós polímers es fa evident en qualsevol de les aplicacions, ja que mentre l'analit d'interès queda retingut selectivament a les cavitats del MIP, en el cas del NIP l'analit queda retingut mitjançant interaccions no específiques o bé pot ser que quedi retingut en un percentatge molt baix. D'aquesta manera el polímer de control permet comparar el comportament de dos polímers semblants estructuralment i evidenciar l'efecte d'empremta molecular del MIP.

1.2.3 Procediment d'extracció en fase sòlida mitjançant polímers amb empremta molecular

Com s'ha comentat anteriorment, els MIPs presenten nombroses aplicacions, però en els darrers deu anys, el seu ús com a sorbents en processos d'extracció en fase sòlida (MISPE) ha estat sens dubte el que més interès ha generat i el nombre creixent de publicacions així ho confirma.

L'ús de MIPs com a sorbents en SPE permet extreure selectivament l'analit(s) d'interès, ja que aquest queda retingut al MIP mentre que la matriu de la mostra i altres substàncies que puguin interferir en la quantificació de l'analit són eliminades. D'aquesta manera s'aconsegueix la selectivitat que fins al moment amb els sorbents convencionals no havia estat possible d'assolir.

Quan els MIPs, i en general qualsevol sorbent que se sintetitza per primer cop, han de ser emprats per l'SPE, és necessari realitzar una avaluació preliminar del polímer abans de dur a terme la seva aplicació com a sorbents degut a que a priori es desconeix quin serà el seu comportament com a materials d'extracció. Per aquest motiu, abans de descriure el procés d'extracció mitjançant MIPs, s'inclou un apartat on s'explica en què consisteix l'avaluació preliminar d'un MIP i el tipus d'informació que es pot obtenir.

1.2.3.1 Avaluació preliminar dels polímers amb empremta molecular

Els mètodes que s'utilitzen habitualment per a avaluar les propietats d'un MIP són l'avaluació cromatogràfica i els experiments d'enllaç tipus discontinu (*batch rebinding analysis*) [49,176,177]. Tots aquests mètodes permeten obtenir una idea sobre com d'empremtat està el MIP, la selectivitat que mostra per l'analit d'interès i també la selectivitat que presenta per altres compostos d'estructura similar.

a) Avaluació cromatogràfica

L'avaluació cromatogràfica d'un MIP permet conèixer el seu factor de capacitat (k') i el factor d'empremta molecular (IF) que s'obtenen a partir dels temps de retenció del *template* (t_t) i del solvent emprat per determinar el volum mort de la columna (t_o).

$$k' = \frac{(t_t - t_o)}{t_o} \qquad\qquad IF = \frac{k'_{MIP}}{k'_{NIP}}$$

k'_{MIP}: factor de capacitat de l'analit al MIP
k'_{NIP}: factor de capacitat de l'analit al NIP

Tant el *template* com el solvent s'injecten en una columna cromatogràfica, la qual s'ha omplert prèviament amb el MIP, sempre i quan el MIP no hagi estat sintetitzat via *in situ*. Així doncs, com més gran sigui la interacció entre la molècula *template* i el MIP, més gran serà el temps de retenció i per tant augmentarà el valor de k'. Aquest valor, cal comparar-lo amb el que s'obté d'injectar el *template* en la columna que conté el polímer de control (NIP). En aquest polímer el *template* queda menys retingut en no presentar cavitats específiques i per tant el temps de retenció serà inferior. Una altra diferència que s'observa entre la columna que conté el MIP i el NIP, és el perfil d'elució del *template*, ja que en el cas del MIP, el pic cromatogràfic acostuma a ser ample, amb llargues cues i asimètric degut a la gran retenció del *template* en aquest polímer i a la heterogeneïtat de les cavitats moleculars. Aquest efecte es veu minimitzat en el polímer de control degut a la manca de les cavitats d'enllaç. De la relació dels factors de capacitat al MIP i al NIP s'obté el factor d'empremta molecular que serà més gran com més gran sigui la diferència entre k'.

L'avaluació cromatogràfica també permet conèixer la selectivitat que presenta el MIP per altres compostos d'estructura semblant a la de la molècula *template*. Mitjançant l'índex de retenció normalitzat (RI), es pot avaluar el grau de reconeixement que presenta el MIP per un analit determinat [154,178]. La molècula *template* presenta un valor de RI igual a1 per definició i la resta de compostos injectats al MIP haurien de donar un valor de RI inferior, ja que idealment haurien de quedar menys retinguts al MIP. Si els RI que s'obtenen tenen valors propers o superiors a 1, llavors es pot considerar que el MIP presenta reactivitat creuada per aquell compost.

$$RI = \frac{(k'\,\text{analit}_{MIP} / k'\,\text{analit}_{NIP})}{(k'\,\text{template}_{MIP} / k'\,\text{template}_{NIP})}$$

Totes aquestes anàlisis cromatogràfiques es duen a terme emprant com a fase mòbil un solvent orgànic, que en alguns casos és el mateix solvent que s'ha utilitzat com a porogen durant la síntesi del MIP, ja que en aquest medi les interaccions que es donen entre el *template* i el MIP haurien d'estar més afavorides degut a que es reprodueixen les condicions de síntesi. L'avaluació cromatogràfica d'un MIP proporciona informació de gran utilitat per al procés de MISPE.

b) Batch rebinding analysis

L'altre tipus d'assaig que es pot realitzar és el que es coneix com anàlisi en discontinu o *batch analysis* [88,97,141,179-181] que té lloc en condicions d'equilibri. En aquest cas, una quantitat de polímer s'afegeix a un vial que conté un solvent orgànic i a continuació s'addiciona una petita quantitat del *template*. Un cop el sistema arriba a l'equilibri, es mesura la quantitat de *template* que queda lliure en solució i per diferència s'obté la quantitat que ha quedat retinguda al MIP. Aquest experiment determina la constant d'enllaç del MIP tenint en compte totes les cavitats que hi ha a la matriu polimèrica. No obstant, la sensibilitat obtinguda pel mètode cromatogràfic en injectar petites quantitats de *template* en llargues columnes analítiques, es perd quan es realitzen les anàlisis en discontinu degut a que en aquest cas només es dóna un únic equilibri. Per tal d'augmentar aquesta sensibilitat i poder observar petites diferències en l'enllaç o bé enllaçar petites quantitats de *template*, es poden emprar altres mètodes com l'assaig amb radiolligands, ja que permeten assolir límits de detecció més baixos. Amb aquest mètode s'avalua el nombre d'empremtes moleculars (cavitats) que presenten una major capacitat d'enllaç i es pot portar a terme de dues maneres diferents, o bé mesurant el nombre de lligands que s'enllacen directament amb el MIP o bé mitjançant un assaig competitiu, on el lligand d'interès competeix per les cavitats d'enllaç amb un altre compost de característiques molt semblants però amb un dels àtoms marcats com radioactiu [49]. El principal inconvenient d'aquesta tècnica és que s'han de manipular materials radioactius, per aquest motiu altres dispositius amb elevada selectivitat com són les reaccions de fluorescència poden ser una alternativa a l'ús d'aquests reactius.

1.2.3.2 Procediment d'extracció en fase sòlida

Un cop avaluat el MIP, el polímer s'introdueix en un dispositiu o en un altre en funció de si l'extracció es duu a terme fora de línia (*off-line*) o bé en línia (*on-line*) amb la posterior tècnica analítica, que en el cas dels sistemes en línia normalment es tracta de la cromatografia de líquids degut a que l'acoblament a altres tècniques analítiques com la cromatografia de gasos (GC) o l'electroforesi capil·lar (CE) és més complex i a més a més, en el cas de la GC existeix una altra limitació pel que fa al solvent d'elució a emprar.

En el cas de processos d'extracció fora de línia, les partícules del MIP s'introdueixen en xeringues de polietilè o de vidre que poden ser de diversos volums. La quantitat de sorbent emprada acostuma a ser entre uns 25-500 mg i està continguda entre mig de dos fritats. Pel que fa als sistemes d'extracció en línia, el dispositiu emprat és diferent ja que el polímer s'introdueix en columnes d'uns pocs mil·límetres de longitud (entre 5-10 mm) i diàmetre (entre 1-3 mm), normalment d'acer inoxidable, i la quantitat de polímer emprat acostuma a ser inferior a 40 mg.

El procés de MISPE consta de tres etapes com qualsevol procés d'extracció en fase sòlida amb sorbents convencionals. No obstant, les característiques que presenten els MIPs fan que hi hagi diferències notables en cadascuna de les etapes. Així doncs, en la primera etapa, d'acondicionament, té lloc l'activació de les cavitats d'enllaç del MIP per tal de potenciar l'efecte d'empremta molecular. A continuació té lloc el pas de la mostra i finalment l'elució. Pel que fa a l'etapa de pas de mostra, cal remarcar que en tractar-se d'un sorbent que presenta selectivitat, permet en alguns casos eliminar la matriu de la mostra durant aquesta etapa, de manera que només l'analit d'interès queda retingut selectivament al MIP [179,182,183]. A mode d'exemple, a la Figura 1.11 es mostra el procés de retenció dels analits en un dispositiu fora de línia quan la mostra s'aplica en un solvent orgànic, normalment apròtic ja que d'aquesta manera s'evita el trencament de les interaccions selectives que s'estableixen entre l'analit i el MIP, ja que acostumen a ser de tipus no covalent. En canvi, quan la mostra que s'aplica és de naturalesa aquosa, llavors els analits acostumen a quedar retinguts en el MIP mitjançant interaccions hidrofòbiques (no específiques) ocupant no només les cavitats d'enllaç del MIP sinó tot el polímer tal i com es pot veure a la Figura 1.11. Com a conseqüència, la matriu de la mostra també queda retinguda i en aquest cas, per tal d'aconseguir una extracció selectiva, cal realitzar una etapa de neteja (*clean-up*) amb un solvent orgànic abans de l'elució dels analits. Cal remarcar que quan la mostra s'aplica en un solvent orgànic, a vegades també és necessària l'etapa de neteja abans de l'elució sempre i quan no s'hagi aconseguit eliminar la matèria orgànica de la mostra durant l'etapa de pas de mostra pel MIP.

L'etapa de neteja és clau per tal d'assolir una extracció selectiva. Per tant, és molt important optimitzar no només el tipus de solvent a emprar, sinó també variables com el volum i el pH, per tal d'explotar al màxim l'habilitat del MIP per reconèixer a l'analit d'interès i eliminar la matriu de la mostra que durant l'etapa de pas de mostra ha quedat retinguda al MIP mitjançant interaccions no específiques. Els solvents que normalment s'utilitzen en l'etapa de neteja acostumen a ser orgànics de baixa polaritat, com per exemple el cloroform, el diclorometà, o el toluè ja que d'aquesta manera s'impedeix que puguin trencar-se les interaccions selectives. A l'hora de triar un solvent o un altre, molts autors es decanten per l'ús del mateix solvent que es va emprar com a porogen durant la síntesi del MIP, ja que el MIP presenta memòria i per tant, en aquestes condicions es reprodueixen les interaccions entre l'analit i el monòmer funcional [93,160,184]. No obstant, en diversos estudis s'ha demostrat que també és possible utilitzar mescles de solvents de diferent polaritat o bé solvents de naturalesa polar (acetonitril, metanol, mescles de solvents orgànics amb aigua, etc...) [163] per tal d'eliminar la matriu de la mostra i no trencar les interaccions selectives que es donen.

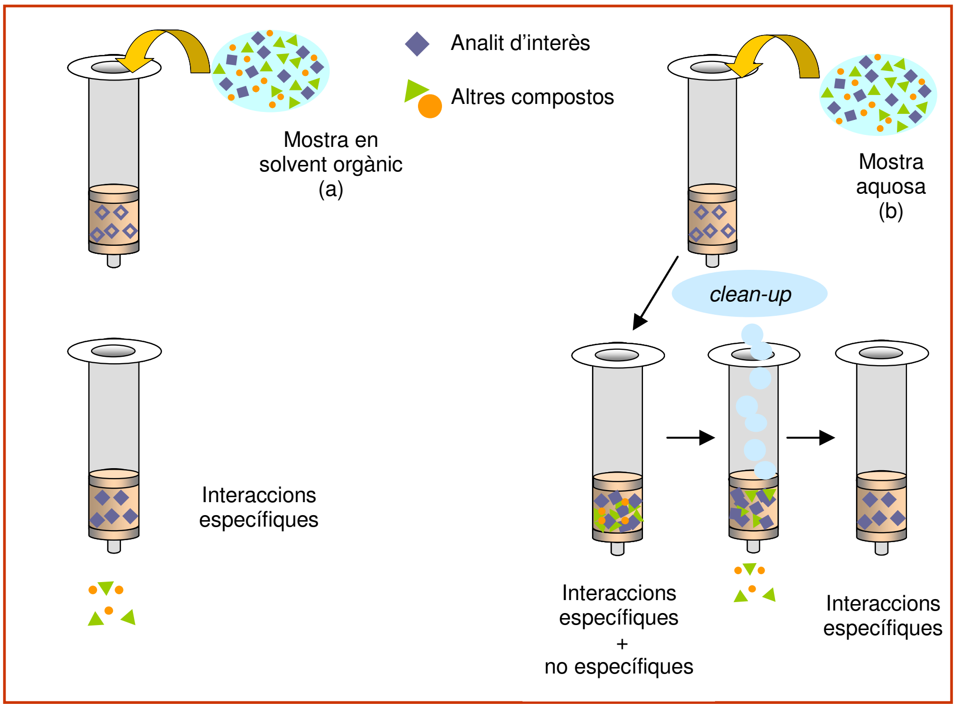


Figura 1.11 Procés de retenció de l'analit d'interès en el MIP quan la mostra s'aplica en un solvent orgànic (a) i quan la mostra és aquosa (b).

Un altre aspecte que cal tenir en compte quan s'aplica una mostra aquosa a través del MIP és l'assecat del polímer abans de realitzar l'etapa de neteja, ja que en alguns casos, s'ha observat com la presència d'aigua produeix problemes de miscibilitat amb el solvent de neteja a emprar i altres vegades les restes d'aigua de la mostra que queden al sorbent actuen com a competidors; tant en un cas com en l'altre, l'eficàcia de la neteja es veu minimitzada i per tant disminueix la recuperació pel compost retingut. Aquest problema pot solucionar-se mitjançant l'assecat del MIP amb una bomba de buit fins que l'aigua s'elimina i el cartutx queda completament sec [184]. No obstant, quan la MISPE és en línia, l'assecat de la precolumna és més complicat i normalment aquesta etapa d'assecat no es realitza. Tot i així, s'han publicat diversos estudis que demostren que quan es treballa en sistemes en línia i no es realitza l'etapa d'assecat prèvia a la de neteja també és possible obtenir una bona selectivitat [97,154,185,186].

Com s'ha comentat anteriorment, alguns dels MIPs emprats en SPE presenten reactivitat creuada fins i tot després de l'etapa de neteja [95,98,181,187-190]. En aquesta situació, el MIP és capaç de retenir selectivament una família de compostos semblants estructuralment, tot i que el polímer hagi estat sintetitzat emprant només a un dels compostos de la família com a molècula *template*. A mode d'exemple, es citen diversos estudis on aquesta característica ha estat aprofitada per a l'extracció d'herbicides com triazines en aigua de riu [93,94,147,160,163,182,188]. Tot i que la majoria de treballs on s'ha observat que el MIP mostrava reactivitat creuada han estat aplicats a l'extracció de compostos en mostres ambientals, darrerament també s'ha aprofitat aquesta característica per a l'extracció de compostos com propranolol [98], verapamil (substància per tractar la hipertensió i l'arrítmia) [181], clenbuterol [189,190], cafeïna [95] i els respectius anàlegs de mostres biològiques com plasma, orina o teixit d'animal destinat al consum humà.

Tot i la importància de realitzar una etapa de neteja adient quan es treballa amb mostres aquoses, en algunes ocasions s'ha demostrat que aquesta neteja es pot evitar quan és possible eluir selectivament l'analit d'interès. A l'igual que en l'etapa de neteja, el solvent d'elució a emprar també ha de ser optimitzat de manera que elueixi selectivament i quantitativament l'analit d'interès però no la resta de matèria retinguda al MIP durant l'etapa de pas de mostra. Aquest solvent d'elució acostuma a ser orgànic de característiques polars i pròtic de manera que sigui capaç de trencar les interaccions selectives (que normalment són electrostàtiques, d'enllaç d'hidrogen etc...) entre l'analit i els grups funcionals del MIP. El solvent d'elució òptim ha de ser capaç d'eluir els compostos de manera efectiva en el mínim volum possible, ja que a mesura que augmenta el volum del solvent d'elució disminueix el factor de preconcentració. Quan el solvent orgànic per si sol no és capaç de trencar les interaccions entre l'analit i el MIP, llavors s'utilitzen barreges entre un solvent orgànic i un modificador com pot ser l'àcid acètic o la piridina. D'aquesta manera el modificador competeix amb els grups funcionals del polímer per enllaçar-se amb l'analit i per tant afavoreix a que les interaccions entre l'analit i el MIP es trenquin amb més facilitat. Una alternativa a aquestes barreges són les mescles de solvents orgànics o bé mescles entre solvent orgànic i aigua. L'avantatge que suposa poder realitzar una elució selectiva de l'analit d'interès és que el sistema d'extracció MISPE pot acoblar-se directament al detector i per tant fa innecessari l'ús d'una tècnica de separació [90,141,191-194].

Sellergren va ser pioner en acoblar directament el procés de MISPE a un detector [191]. En aquest estudi, la síntesi del MIP es va dur *in situ* en un tub de vidre de dimensions inicials 150 mm x 3 mm i.d. El *template* utilitzat va ser la pentamidina i després del procés de polimerització, el tub de vidre es va tallar i es van col·locar els fritats corresponents per a que pogués ser connectat a un cromatògraf de líquids senzill. Degut a problemes de pressió, els tubs es van tallar de manera que les dimensions finals de la columna van ser de 100 mm x 3 mm i.d. El MIP obtingut es va emprar per a l'extracció selectiva de la molècula *template* en mostres d'orina humana. Després de diverses dilucions de la mostra amb tampons i solvent orgànic, 100 ml de la solució resultant es van passar a través de la columna i a continuació es van realitzar diverses neteges amb acetonitril/fosfat potàssic a pH 9 més 2.5 ml de la mateixa mescla a pH 5. Degut a l'elevada selectivitat del MIP i a una elució eficaç amb una fase mòbil adient, la pentamidina era eluïda directament cap al detector d'UV i quantificada sense presència d'interferents, però la recuperació de la pentamidina va ser només d'un 10%. L'autor justifica aquest resultat com una conseqüència del pH al qual es treballa, on es va comprovar que en aquestes condicions la selectivitat del MIP era màxima tot i que la retenció de la pentamidina fos limitada i per tant la recuperació per l'analit d'interès fos baixa. Aquest fet és freqüent quan es treballa amb MISPE ja que sovint cal arribar a un compromís entre les recuperacions i la selectivitat.

Una de les tècniques que també permet l'acoblament en línia amb el detector sense necessitat d'un sistema de separació és el que van desenvolupar Mullet *et al.* l'any 1998 conegut com sistema d'elució amb pulsos (*pulsed elution* (PE)) [141]. En aquest cas, el MIP empremtat amb teofilina es va empaquetar en una microcolumna de dimensions 80 mm x 4 mm i.d. i es va connectar a un HPLC. La fase mòbil emprada era formada per cloroform i la mostra de sèrum es va injectar en la microcolumna. Es van dur a terme múltiples injeccions de 20 µl fins a introduir un volum total de mostra de 300 µl. Un minut després de les successives injeccions, eluia el pic corresponent al solvent i un altre a la matriu de la mostra i dos minuts després es va portar a terme l'elució de la teofilina amb 20 µl de metanol directament cap al detector d'UV.

La PE és un mètode ràpid de preconcentració que eluix l'analit retingut al MIP de forma efectiva evitant el problema de perfils d'elució amb llargues cues que donen alguns materials empremtats en retenir fortament el compost d'interès. A més a més, les mides de la microcolumna eviten l'eixamplament de pics i la poca quantitat de sorbent emprada afavoreix també que l'elució de l'analit sigui efectiva. No obstant, aquesta tècnica va ser modificada posteriorment per tal d'afavorir la complerta eliminació de la matriu de la mostra. Així doncs, Mullet et al. l'any 1999 van desenvolupar per primera vegada l'elució diferencial amb pulsos (*Differential Pulsed Elution* (DPE)) [192] la qual es diferenciava de l'anterior en la introducció d'una etapa de neteja després del pas de la mostra i abans de l'elució de l'analit d'interès. Hi ha diversos estudis publicats fins al moment on la tècnica emprada per a l'extracció en línia de compostos ha estat la DPE [90,192-196]. En aquests treballs, compostos com nicotina, teofilina, cefalexina i 4-aminopiridina s'extreien de mostres biològiques com plasma i sèrum, a excepció de la nicotina que es determinava en tabac, mitjançant l'ús d'aquesta tècnica de preconcentració. El sistema emprat era el mateix que el descrit per la PE, només que ara la microcolumna presentava unes dimensions diferents, les quals variaven entre 35-80 mm x 0.8 mm i.d. L'etapa de neteja es va portar a terme mitjançant diverses injeccions, normalment de 20 µl, d'un solvent capaç de trencar només les interaccions no específiques dels analits retinguts. És d'esperar que si l'analit queda retingut per la suma de les interaccions específiques més les no específiques en realitzar l'etapa de neteja disminuiexi la recuperació del compost en comparació amb la trobada en el mètode anterior de PE [192].

Aconseguir una elució selectiva dels analits retinguts al MIP representa un gran avantatge, ja que permet l'acoblament directe entre el MIP i el detector i per tant s'aconsegueixen temps d'anàlisi més curts. No obstant, no sempre és possible eluir selectivament l'analit d'interès. Quan això succeeix, llavors és necessari l'ús d'una tècnica de separació (normalment es tracta de la cromatografia de líquids) prèvia al detector, ja sigui treballant en mode fora de línia com en línia. Pel que fa al sistema d'elució en línia l'etapa d'elució és la que presenta més limitacions ja que, com es pot veure a la Figura 1.12, el solvent d'elució del(s) analit(s) retingut(s) al MIP és la pròpia fase mòbil del sistema cromatogràfic [89,91,148,167,197-199] que sovint no és el solvent apropiat per trencar les interaccions selectives entre el *template* i el MIP. Això implica que o bé s'afegeixin modificadors a la fase mòbil en proporcions elevades com per trencar les interaccions, però que aleshores podrien deteriorar la columna analítica, o bé es canviï la fase mòbil del sistema, tenint en compte que l'adient per trencar les interaccions pot ser que no sigui l'adequada per a la separació cromatogràfica dels compostos. Si el sistema emprat és el de MISPE fora de línia, aquests inconvenients són fàcilment solucionats.

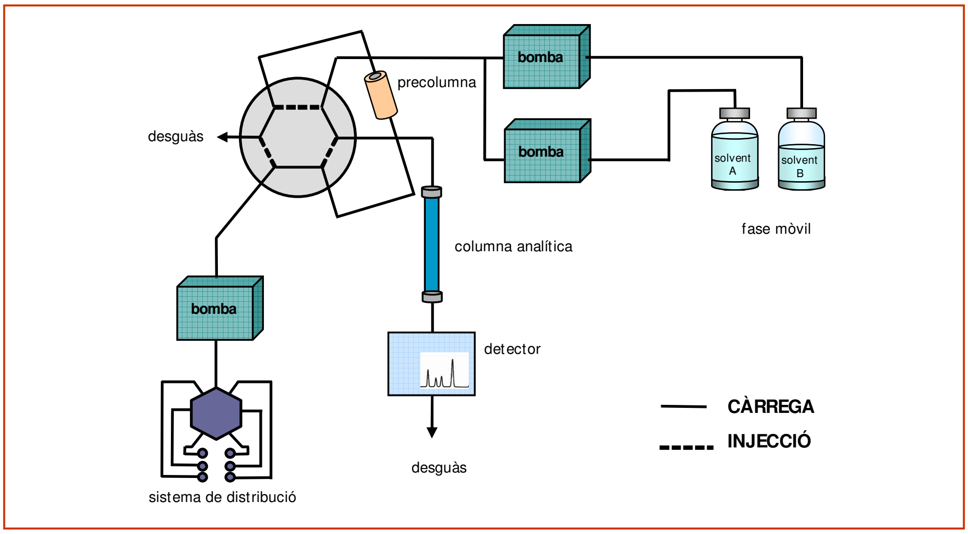


Figura 1.12 Sistema d'extracció en línia.

Un cop revisats els trets característics del procediment d'extracció en fase sòlida emprant MIPs, a continuació s'inclou un apartat on es descriuen diversos exemples dels treballs realitzats fins ara sobre l'aplicació de la MISPE a l'extracció de compostos en mostres ambientals i biològiques.

1.2.4 Aplicacions de polímers amb empremta molecular com a sorbents a l'extracció en fase sòlida

L'SPE emprant MIPs com a sorbents ha estat aplicada a l'extracció de compostos en diversos tipus de mostra; els més habituals són el mediambiental i el biològic, sent aquest últim el més nombrós ja que aquests tipus de mostres són de matriu complexa i realitzar extraccions amb elevada selectivitat suposa un gran avantatge. En els darrers anys també s'han dut a terme aplicacions a l'extracció de compostos en mostres de matriu més complexa com són les mostres sòlides, ja siguin sediments, teixits animals (fetge, ronyó, etc...) o bé aliments (vi, sucs, blat, llevat, etc...).

Fins al moment, els treballs publicats en MISPE fora de línia són els més nombrosos, degut principalment a la simplicitat en la instrumentació requerida i a la flexibilitat que aquests sistemes ofereixen pel que fa a la quantitat de sorbent i de solvent d'elució a utilitzar. En alguns estudis, l'optimització del procés de MISPE s'ha portat a terme en sistemes fora de línia mentre que quan ha estat possible per qüestions de compatibilitat entre el solvent d'elució i fase mòbil, la posterior aplicació a l'extracció de compostos s'ha dut a terme emprant un sistema en línia amb una tècnica cromatogràfica.

Pel que fa al nombre de treballs de MISPE fora de línia aplicats a l'extracció de compostos en mostres ambientals (aigua de riu, aigua de llacs, aigua de mar, aigües residuals i sediments) cal assenyalar que ha experimentat un augment considerable en els últims anys [93,96,160,163,187,188, 200-205]. El primer estudi d'aquest tipus, va ser desenvolupat per Ferrer *et al.* l'any 2000 [93]. En aquest treball, es va sintetitzar un MIP amb el pesticida terbutilazina com a *template* i degut a que aquest MIP presentava reactivitat creuada, després de realitzar l'etapa de neteja amb diclorometà, es va extreure una barreja de sis clorotriazines de 100 ml d'aigua de riu. Es van obtenir molt bones recuperacions per a tots el compostos (>80%) excepte per a un d'ells, la propazina, en que la recuperació no va ser superior al 55%.

Altres autors com Chapius [96], Pap [160] i Turiel [188] també van sintetitzar MIPs per a l'extracció de triazines. Tots tres MIPs presentaven reactivitat creuada per a un grup de triazines com en el cas del treball descrit prèviament per Ferrer *et al.*

Els MIPs sintetitzats per Chapius i Pap es van preparar seguint la mateixa metodologia descrita per Ferrer, per tant era d'esperar que també presentessin reactivitat creuada. No obstant, Turiel va emprar una altra triazina com a *template* (propazina), va canviar de porogen (toluè enlloc de diclorometà) i va realitzar la polimerització tèrmicament, però tot i així, el MIP resultant també mostrava reactivitat creuada. Per tal de confirmar que en tots els casos el MIP retenia al grup de triazines mitjançant interaccions selectives es van comparar aquests resultats amb els obtinguts en realitzar el mateix experiment en el polímer de control, on els compostos s'eluien durant l'etapa de neteja.

No obstant, pel que fa al procés d'extracció, cadascun d'aquests autors va desenvolupar mètodes diferents. En el cas de Chapius, a l'igual que Ferrer, la mostra aquosa es va aplicar directament sobre el MIP. Com que l'etapa de neteja es portava a terme amb un solvent immiscible amb aigua (diclorometà), Chapius va introduir una etapa d'assecat del sorbent abans de netejar-lo amb el solvent orgànic. Es va observar que les recuperacions per les triazines estudiades disminuïen molt quan s'extreien de mostres reals (<20%), fet que no succeïa quan l'extracció es realitzava en aigua Milli-Q. Aquesta disminució en les recuperacions la van justificar per la presència dels cations Na^+ i Ca^{2+} que conté la matriu de la mostra i principalment pel Ca^{2+} que durant l'etapa de pas de la mostra pel MIP s'intercanvia pel protó de l'àcid carboxílic que hi ha a l'estructura de l'àcid metacrílic del MIP (emprat com a monòmer funcional durant la síntesi del MIP). Per tal de solucionar aquest problema, després de l'etapa de pas de mostra (50 ml), el MIP es va rentar amb HCl (0.1 M) i aigua Milli-Q, de manera que les cavitats del MIP es regeneraven. A continuació el MIP s'assecava i es portava a terme l'etapa de neteja amb el solvent orgànic. Les recuperacions pel grup de triazines van augmentar considerablement ja que van ser superiors al 89%.

Aquest MIP també es va aplicar a l'extracció de compostos polars que provenen de la degradació de les triazines. Degut a que aquests compostos presentaven baixos volums de ruptura amb el MIP (50 ml) i baixes recuperacions (no superiors al 65%), Chapius va utilitzar un sorbent de mode mixt, on 100 mg de PS-DVB (sorbent polimèric comercial amb elevada àrea superficial, 1080 m^2/g, Mallinckrodt

Baker) es combinava amb 200 mg del MIP per a l'extracció d'aquests compostos. La millora en la recuperació va ser notable ja que permetia preconcentrar fins a 100 ml de mostra amb recuperacions superiors al 85%.

Pap i Turiel, tot i sintetitzar també un MIP per a l'extracció selectiva dels mateixos plaguicides (triazines) no van aplicar directament la mostra aquosa sobre el MIP. En l'estudi de Pap [160], es va acoblar un disc de C_{18} al cartutx amb el MIP. Aquest sistema va permetre preconcentrar fins a 500 ml d'aigua de riu, la qual passava primer pel disc de C_{18} i després d'una etapa d'assecat, els analits i altres substàncies retingudes en aquest dispositiu s'eluien amb 30 ml de diclorometà directament cap al cartutx amb el MIP, on només tenia lloc la retenció selectiva dels analits ja que la resta de compostos eren eluïts durant aquesta etapa. Per portar a terme l'elució dels analits retinguts selectivament al MIP es desconnectaven els dos dispositius d'extracció.

D'una manera semblant, Turiel [188] va preconcentrar primer la mostra (500 ml d'aigua de l'aixeta) a través de dos discos d'extracció de PS-DVB 3M (Empore). L'elució de la mostra es realitzà amb acetonitril i posteriorment aquest solvent va ser evaporat per reconstituir la mostra en 1 ml de toluè i aplicar-la a través del MIP. El toluè va ser el solvent triat degut a que la síntesi del MIP s'havia realitzat amb aquest com a porogen. Després d'aquesta etapa d'extracció, l'eluat final es va analitzar per cromatografia miscel·lar electrocinètica.

L'estudi de la MISPE aplicada a l'extracció de triazines en mostres ambientals ha generat gran interès. No obstant, altres compostos com herbicides tipus clorofenoxiàcids [163], sulfonilurees i fenilurees [187,204], ions com el cianur [200], hidrocarburs aromàtics policíclics (PAHs) com el benzo[a]pirè [201] i organoestànnics [205] també han estat extrets de mostres mediambientals. En tots aquests treballs (excepte el desenvolupat per Martin-Esteban *et al.* [204], on 100 ml de d'aigua superficial es feia passar primer a través d'un disc d'extracció de PS-DVB 3M Empore) la mostra aquosa ha estat aplicada directament en el cartutx d'extracció que conté el MIP i s'han obtingut bones recuperacions, fins i tot després de preconcentrar grans volums de mostra (1000 ml) [187,205]. Aquests dos treballs són dels pocs que s'ha realitzat fins ara en l'àmbit mediambiental on un volum de mostra tan gran ha estat preconcentrat amb èxit. Així doncs, en l'estudi realitzat per Zhu *et al.* [187] es va preconcentrar 1000 ml d'aigua de l'aixeta, aigua superficial i de pluja per a l'extracció selectiva d'una mescla de sulfonilurees (grup d'herbicides) amb recuperacions superiors al 96%. Aquest MIP es va preparar amb metsulfuron-metil com a *template* i degut a la reactivitat creuada que presentava després de l'etapa de neteja amb el corresponent solvent orgànic (diclorometà/acetonitril (93:7)), va ser possible extreure una barreja de cinc sulfonilurees.

Darrerament, Gallego-Gallegos *et al.* [205] van preparar diversos MIPs variant algunes de les condicions experimentals com el monòmer funcional (metacrilat sòdic o 4-VP) i el porogen (metanol/aigua (3:1), acetonitril o toluè) per tal de trobar el que presentés millor reconeixement per la molècula *template* (tributilestany). El MIP preparat amb metacrilat sòdic i acetonitril va resultar el més adient per a l'extracció d'aquest compost en 1000 ml d'aigua de mar. Després de realitzar l'etapa de neteja amb 10 ml d'aigua (per tal d'eliminar l'excés de sal) i 6 ml del corresponent solvent orgànic (metanol/diclorometà (4:1)), la recuperació pel tributilestany va ser d'un 93%.

La MISPE també ha estat aplicada a l'extracció de compostos mediambientals en sediments tot i que el nombre de treballs existents és reduït [93,188,202-204]. Alguns d'aquests estudis han estat descrits prèviament com són el de Ferrer [93], el de Turiel [188] i el de Martin-Esteban [204] on després de fer el tractament adient a la mostra, l'extracte que s'obté se sotmet al mateix procediment de MISPE descrit per a mostres aquoses. En els altres dos treballs [202,203], la MISPE ha estat desenvolupada específicament per a l'extracció de triazines i monosulfuron, respectivament, en aquest tipus de mostra. Tots dos presenten característiques comunes com són el pretractament de la mostra, tot i que empren tècniques diferents, i l'aplicació de l'extracte resultant, prèviament evaporat i reconstituït en el solvent adient, aplicat a través del MIP. En l'estudi desenvolupat per Chapius [202] en què el MIP és sintetitzat emprant ametrina com a *template*, es compara el resultat que s'obté quan s'empra aquest MIP amb el que s'obté quan s'utilitzen ISs com a materials d'extracció i s'observa que la selectivitat obtinguda emprant tots dos tipus de sorbents és comparable.

Tot i l'augment que s'ha produït en els darrers anys en el nombre de publicacions de MISPE en el camp mediambiental, l'aplicació d'aquesta tècnica a l'extracció de compostos en mostres biològiques continua sent la més nombrosa. Fluids biològics com el plasma, el sèrum i l'orina són les mostres més estudiades [84-86,88,95,190,206-210], tot i que darrerament també s'han publicat alguns treballs que fan referència a l'extracció de compostos en mostres més complexes com són els teixits d'animals [149,189,211,212].

En el cas dels fluids biològics, la mostra pot ser aplicada directament al cartutx d'extracció després d'una etapa de filtració prèvia, però en la majoria d'estudis es realitza un tractament previ a la mostra que consisteix en una dilució de la mateixa amb un solvent orgànic per precipitar les proteïnes presents en aquest tipus de mostra i per disminuir el contingut d'aigua de la mostra [84,88,95,190,208]. Els MIPs sintetitzats amb aquest objectiu han estat empremtats amb molècules tan diverses com el propranolol, la cafeïna, el clenbuterol i la sameridina modificada amb un grup metil (compost emprat com a anàleg de la molècula objectiu, sameridina, per tal d'evitar els problemes de sagnat del MIP) [84]. Aquest darrer treball ha estat de tots els esmentats, l'únic on el sistema analític emprat després de la MISPE va ser la cromatografia de gasos ja que en la resta de treballs, la cromatografia de líquids ha estat la tècnica de separació seleccionada. En aquest estudi desenvolupat per Andersson *et al.* es va dur a terme la comparació dels cromatogrames obtinguts després de realitzar l'extracció de la sameridina en mostres de plasma a través del MIP i mitjançant un procés d'extracció líquid-líquid. La diferència entre els dos mètodes d'extracció era notable ja que els cromatogrames obtinguts després de preconcentrar la mostra a través del MIP presentaven menys pics corresponents a la matriu de la mostra.

No obstant, sobretot en els darrers anys, s'han desenvolupat estudis on els biofluid s'han aplicat directament al MIP [82,85,206,209]. En aquests estudis, tot i que el volum de mostra preconcentrat és de pocs mil·lilitres, l'etapa de neteja abans de l'elució també és clau per eliminar les interferències que provenen de la matriu, tal i com van demostrar Xie [206] i Chassaing [209] en els seus treballs, on s'extreia de mostres de plasma compostos com quercitina i un compost desenvolupat per l'empresa farmacèutica Pfiezer (de nom desconegut per motius de confidencialitat), respectivament. Ambdós autors van comparar els procés de MISPE amb el resultat de l'extracció emprant sorbents convencionals (C_{18}) i en tots dos treballs la millora pel que fa a l'eliminació de matèria orgànica era notable en els procés de MISPE.

Els treballs on s'ha aplicat el MIP a l'extracció de compostos en mostres de teixit animal són encara una minoria, degut principalment a la complexitat d'aquest tipus de mostra. Per aquest motiu, el pretractament que requereixen aquestes mostres acostuma a ser més laboriós (trituració, homogenització, centrifugació, etc...). El primer estudi realitzat en aquest àmbit va ser publicat per Muldoon *et. al* [149]. En aquest cas el MIP va ser empremtat amb atrazina i es va aplicar a l'extracció d'aquest compost en mostres de teixit animal (fetge boví). L'extracte obtingut després de realitzar el tractament al teixit es va aplicar a través del cartutx amb el MIP i es va aconseguir una neteja de la matriu de la mostra molt efectiva i per tant facilitava la quantificació de l'analit d'interès amb la posterior tècnica analítica (HPLC o assaig amb enzim unit a immunosorbent (ELISA)), la qual cosa demostra que la tècnica de MISPE es pot aplicar a aquest tipus de mostra.

Un altre exemple recent, d'aplicació directa al MIP de l'extracte obtingut després del tractament del teixit, és el treball desenvolupat per Kootstra *et al.* [212]. En aquest cas, es va extreure una mescla de 8 fàrmacs broncodilatadors, de múscul boví. El MIP emprat en aquest estudi havia estat preparat amb clenbuterol com a *template*, però no va ser sintetitzat per l'autor, sinó que es tractava d'un MIP comercial (MIP4SPE, MIP Technologies, Lund, Suècia). El mètode desenvolupat per a l'extracció i quantificació d'aquests compostos (MISPE i posterior anàlisi amb LC-MS-MS) es va validar i els bons resultats que es van obtenir no només per teixit d'aquest animal sinó també per a d'altres com conill, ànec i gall d'indi demostren que la combinació de MIPs amb LC-MS té un gran potencial en aquest camp.

No obstant, quan es treballa amb aquest tipus de mostres, tot i el tractament previ al qual se sotmet el teixit abans de ser aplicat a través del MIP, acostumen a quedar restes de proteïnes i greixos, els quals poden donar lloc a problemes d'obstrucció de les cavitats del MIP, i per tant a una disminució del reconeixement del compost i de la seva recuperació. Per tal de minimitzar aquests inconvenients, Brambilla *et al.* [189] i Crescenzi *et al.* [211] van desenvolupar altres mètodes d'extracció basats en l'ús de dues etapes d'extracció, la primera que té lloc a través d'un sorbent comercial i la segona a través del MIP. Així doncs, en el cas de Brambilla *et al.*, la mostra (fetge boví) es va fer passar a través d'un cartutx que contenia Extrelut 20, on quedaven retingudes la majoria d'impureses. L'extracte recollit d'aquesta primera elució del sorbent comercial es va fer passar pel MIP i com a conseqüència l'analit d'interès (clenbuterol) es va poder quantificar ja que la major part de les interferències es van poder eliminar. Aquest mètode d'extracció no només permet fer una neteja més exhaustiva sinó que també permet fer un canvi del medi de la mostra de manera que quan aquesta passi pel MIP, l'analit d'interès pugui ser preconcentrat en el solvent òptim.

Crescenzi *et al.* [211] van proposar un mètode d'extracció una mica diferent, ja que a més de netejar la mostra també minimitzava molt el seu pretractament. Així doncs, el teixit (fetge boví) era triturat i barrejat amb uns mil·ligrams d'un sorbent comercial com per exemple C_{18}. Aquesta mescla va ser introduïda en una xeringa d'extracció buida i amb un solvent orgànic es va eluir l'analit. L'extracte obtingut d'aquesta primera extracció es va passar a continuació pel cartutx del MIP on l'analit quedava retingut selectivament. És important senyalar, que en aquest cas, el MIP havia estat sintetitzat emprant com a *template* bromoclenbuterol, anàleg de la molècula d'interès. Mitjançant aquest mètode, l'extracte final que es va obtenir era molt net, i en aquest estudi, es va evaporar i redissoldre en el solvent apropiat per a ser injectat en un sistema d'HPLC amb detector electroquímic (ECD) o bé espectròmetre de masses amb trampa d'ions.

Tot i el gran nombre d'estudis desenvolupats en sistemes d'extracció de MISPE fora de línia l'interès per automatitzar els sistemes d'extracció i acoblar-los a la posterior tècnica analítica suposa avantatges importants que han donat lloc a la publicació de diversos treballs on la MISPE ha estat acoblada en línia al posterior sistema analític. Tot i que no són tants els articles publicats emprant sistemes en línia, en els darrers anys han experimentat un augment considerable sobretot en el camp mediambiental. A més a més, els bons resultats obtinguts, posen de manifest que quan el MIP ho permet i es disposa de la instrumentació necessària, és més interessant treballar en línia ja que és possible assolir límits de detecció més baixos.

La primera aplicació en línia d'un MIP a l'extracció d'un compost en aigua de riu va ser duta a terme per Masqué *et al*. [154]. En aquest treball es va sintetitzar un MIP amb 4-nitrofenol (4-NP) com a *template* i en ser aplicat en SPE es va comprovar que el MIP era capaç d'extreure selectivament a aquest compost d'una mescla de compostos fenòlics després d'una etapa de neteja amb uns pocs mil·lilitres de diclorometà. Tot i que la recuperació no era gaire elevada (~40%) el MIP presentava una gran selectivitat pel 4-NP, a més a més de fer disminuir molt la banda dels àcid húmics al començament del cromatograma, la qual cosa feia possible la correcta quantificació de l'analit en qüestió. La precolumna que contenia el MIP es va acoblar a un cromatògraf de líquids amb detector UV. El fet de que el solvent d'elució emprat fos una mescla d'acetonitril/àcid acètic (99:1) i que aquest fos compatible amb la posterior columna analítica va permetre l'ús d'aquest MIP en aquest sistema acoblat.

Altres autors com Watabe [89], San Vicente [185] i Mena [186] també van desenvolupar treballs de MISPE en línia per a l'extracció de compostos en mostres d'aigua. En l'estudi dut a terme per Watabe *et al*. [89] es va utilitzar un sistema en línia, molt semblant al desenvolupat per Masqué, per a l'extracció de bisfenol A en mostres d'aigua a baixos nivells (ppt), tot i que per a la síntesi del MIP el compost emprat com a *template* es tractava d'un anàleg (p-tert-butilfenol). San Vicente *et al*. [185] també van preparar un MIP selectiu per extreure bisfenol A de mostres d'aigua de riu i en aquest cas, el propi analit d'interès va ser emprat com a *template*. Les condicions de síntesi, així com el monòmer funcional i les proporcions a emprar van ser optimitzades en un estudi previ fent ús d'un disseny d'experiments i anàlisi multivariant [213], el qual va permetre trobar el MIP amb les millors característiques de selectivitat i afinitat que van permetre extreure selectivament aquest compost d'entre una mescla de compostos semblants a ell estructuralment.

Per una altra banda, a diferència dels autors citats prèviament, Mena *et al.* [186] van desenvolupar un sistema d'extracció en línia on el MIP es va acoblar directament a un detector voltamperomètric fent innecessari l'ús d'una tècnica de separació degut a l'elevada selectivitat presentada per aquest polímer. En aquest treball es va sintetitzar un MIP selectiu pel pirimicarb (insecticida emprat en agricultura) i es va aplicar a l'extracció d'aquest compost en diferents tipus d'aigua (aixeta, riu, mar) amb bones recuperacions en tots els casos (55-75%).

En tots aquests treballs la mostra aquosa es va aplicar directament sobre el MIP, no obstant, tot i obtenir-se bones recuperacions, Koeber *et. al* van desenvolupar un nou sistema d'extracció per tal d'evitar la presència d'aigua durant el pas de la mostra en el MIP [182]. Per tal d'aconseguir-ho, la mostra (aigua de riu) passava inicialment per una columna RAM que estava acoblada a la columna amb el MIP. D'aquesta manera, s'aconseguia una primera neteja de la matriu de la mostra en el RAM i els analits que quedaven retinguts s'eluien amb acetonitril cap al MIP. El MIP sintetitzat per Koeber havia estat empremtat amb terbutilazina i presentava reactivitat creuada per un grup de triazines, tot i que per alguns dels compostos no s'obtenien recuperacions quantitatives. Així doncs, els analits retinguts al RAM eren preconcentrats al MIP en el solvent orgànic i posteriorment eluïts en línia cap a un cromatògraf de líquids acoblat a un espectròmetre de masses.

L'aplicació de la MISPE en línia a una tècnica analítica també ha estat desenvolupada per a l'extracció de compostos en mostres biològiques. El primer treball realitzat en aquest àmbit va ser publicat per Sellergren l'any 1994 com s'ha comentat prèviament. A banda dels estudis desenvolupats en mode PE i DPE citats anteriorment a l'apartat 1.2.3.2. [90,141,192-196], autors com Andersson [91], Theodoridis [197] i Möller [214] han desenvolupat mètodes d'extracció en línia acoblats a la cromatografia de líquids per a l'extracció de compostos tan diversos com analgèsics [91], cafeïna [197] i retardants de flama [214]. El MIP preparat per

Andersson i el que va preparar Möller, presentaven reactivitat creuada i es va aprofitar per a l'extracció d'una mescla d'analgèsics i d'una mescla de compostos organofosforats retardants de flama, respectivament.

Tal i com s'ha descrit abans per a l'extracció de compostos en mostres biològiques fora de línia, per tal d'evitar que les restes de la matriu després de fer el tractament quedin retingudes al MIP i per tant disminueixi el reconeixement molecular del MIP, sovint es combinen dos sorbents durant l'etapa d'extracció. En aquest cas, el sorbent comercial (RAM o C_{18}) està connectat en línia amb el MIP. A mode d'exemple, es poden citar diversos treballs com el desenvolupat per Mullet *et al.* [97], que van preparar un MIP amb verapamil (compost per tractar l'hipertensió i l'arritmia) com a *template*, i que degut a la reactivitat creuada d'aquest MIP es va poder extreure una mescla de compostos de la mateixa família. La mostra (orina i plasma) es va preconcentrar primer a través d'un RAM on s'eliminaven els compostos macromoleculars. Els analits de baix pes retinguts en aquest sorbent s'eluien cap al MIP i en passar a través d'ell en un solvent orgànic i en absència d'aigua, tenia lloc una retenció selectiva dels compostos d'interès. Finalment, la separació i detecció dels compostos es va dur a terme en un sistema cromatogràfic acoblat a un espectròmetre de masses. De manera semblant a Mullet, Boos *et al.* [199] van desenvolupar un sistema d'extracció RAM-MISPE en línia a la cromatografia de líquids per extreure tramadol de plasma.

Per una altra banda, Bjarnason *et al.* [148] van fer ús d'un sorbent C_{18} abans del MIP per a l'extracció d'una mescla de triazines en mostres d'orina, entre d'altres. Aquest MIP també presentava reactivitat creuada com tots els MIPs descrits fins al moment empremtats amb alguna triazina.

Degut als bons resultats que es van obtenir combinant dos sorbents, Haginaka i Sanbe van empremtar molecularment un sorbent tipus RAM [167,198] per tal d'assolir el mateix resultat però utilitzant només una columna. Aquest nou material va ser aplicat a l'extracció de naproxen [167] i propranolol [198] en plasma, tot i que el MIP utilitzat per a l'extracció de naproxen va ser empremtat amb ibuprofen, un antiinflamatori molt semblant estructuralment, degut al sagnat que presentava el

MIP quan era empremtat amb el propi naproxen. Malgrat l'avantatge d'emprar una sola columna quan s'empremta molecularment el RAM, el pas de la mostra presenta restriccions pel que fa al solvent a emprar, ja que està limitat a tampons aquosos per tal d'evitar la precipitació de les proteïnes de la mostra [97].

Així doncs queda de manifest que el procés d'empremta molecular és una tècnica amb molt de futur, no només perquè ofereix nombroses avantatges respecte altres materials que també presenten selectivitat sinó perquè també permet obtenir sorbents per a l'extracció selectiva d'una gran varietat d'analits tal i com s'ha demostrat al llarg d'aquest apartat.

1.2.5 Noves tendències

En aquest apartat es descriuen tant algunes de les noves tendències dins del camp de la tecnologia d'empremta molecular i de la MISPE com els avanços que han sorgit, dirigits cap a la millora d'aspectes relacionats amb l'obtenció del MIP, i per una altra banda a solucionar algunes de les limitacions que presenta el procés de MISPE descrites al llarg d'aquest capítol.

Pel que fa a la millora en la síntesi de MIPs, diversos aspectes han de ser considerats, com per exemple que el MIP pugui ser obtingut en forma de partícules esfèriques, homogènies, de la mateixa mida i no sigui necessari haver de triturar i tamisar, especialment quan ha de ser emprat en SPE. Poder sintetitzar el polímer dins del propi cartutx d'extracció (*in situ*), oferiria un gran avantatge pel que fa a aquesta aplicació. Autors com Pérez-Moral, en el darrer *International Workshop on MIPs* (Cardiff, 2004) [215], van presentar la síntesi d'un MIP mitjançant polimerització en suspensió en fluorocarbó dins del mateix cartutx d'extracció. La mida i el nombre de les partícules esfèriques obtingudes depenen del temps i/o de la velocitat del microhomogenitzador emprat. Es van preparar dos MIPs, un empremtat molecularment amb propranolol i l'altre amb morfina i un cop obtinguts els MIPs, aquests es rentaven en el mateix cartutx per tal d'eliminar el *template.* Cal remarcar que la mida i les característiques d'enllaç de les partícules produïdes eren semblants a les obtingudes mitjançant una polimerització en suspensió convencional.

Un altre aspecte relacionat amb la síntesi de MIPs que cal millorar és el del sagnat que presenten alguns MIPs. Com ja s'ha comentat a l'apartat 1.2.1. d'aquest capítol, darrerament s'estan aplicant altres tècniques (MAE, extracció amb fluids supercrítics) com a alternativa a la extracció Soxhlet, de manera que permetin l'eliminació de forma efectiva de la molècula *template* un cop sintetitzat el MIP.

Una altra de les fites marcades pel que fa a la síntesi de MIPs, és la de l'ús de macromolècules com pèptids, proteïnes o microorganismes, com a *template*, ja que fins al moment, la majoria d'estudis s'han portat a terme emprant molècules de mida relativament petita. Aquest fet és degut a que aquests tipus de molècules, presenten alguns inconvenients com nombrosos grups funcionals, limitada estabilitat front temperatura i pH i baixa tolerància als mètodes convencionals de síntesi de MIPs. Per aquest motiu seria un repte poder arribar a sintetitzar MIPs utilitzant proteïnes senceres com a molècula *template,* com per exemple l'àcid desoxirribonucleic (ADN). L'aproximació que s'ha fet fins al moment en aquest camp ha estat emprant seqüències de pèptids com a *template* (*epitope approach*), la qual representa un fragment de tota la proteïna, però s'ha comprovat com el MIP resultant ha estat capaç de reconèixer a la proteïna que contenia la part seleccionada com a *template* [216].

En els últims anys també s'han dut a terme estudis teòrics sobre com realitzar la síntesi d'un MIP mitjançant un disseny racional i no per intuïció o per prova i error com s'acostuma a fer habitualment. Degut a que són molts els paràmetres a optimitzar en el procés d'obtenció d'un MIP, la tendència que s'observa darrerament va cada cop més dirigida a la creació de llibreries combinatorials que permeten trobar d'una manera ràpida i senzilla la mescla i les condicions de síntesi per a proporcionar el MIP òptim. Alguns exemples de treballs que fan ús d'aquesta tècnica han estat desenvolupats per Sellergren [127], Cederfur *et al.*

[217] o Dirion *et al.* [218]. En tots ells es preparen mini-MIPs *in situ* (MIPs que s'obtenen a partir de les quantitats mínimes necessàries per tal de poder ser obtinguts), és a dir, en el mateix lloc on després es realitza l'assaig per veure l'efecte d'empremta molecular. Aquest mètode normalment es porta a terme en un dispositiu que conté fins a 96 vials per fer diferents experiments de manera automàtica. Aquesta tècnica permet trobar amb èxit les condicions idònies per tal d'obtenir un MIP amb el menor nombre d'interaccions no específiques.

La majoria de llibreries combinatorials que es creen no contemplen les possibles interaccions entre els diversos factors estudiats perquè es tracta d'una tècnica d'optimització univariant. No obstant, autors com Navarro-Villoslada *et al.* [213] i Baggiani *et al.* [219] proposen l'aplicació de mètodes multivariants (regressió per mínims quadrats parcials (PLS) i anàlisi per components principals (PCA), respectivament) que permeten trobar el MIP òptim tenint en compte en tot moment la interrelació que existeixen entre els diversos paràmetres. Els models multivariants demostren que hi ha una correlació molt gran entre els paràmetres seleccionats a optimitzar en el disseny experimental i les cavitats d'enllaç resultants. Tenint en compte la gran quantitat de paràmetres que cal controlar perquè influencien a la selectivitat, afinitat, capacitat etc. del MIP, aquest mètode quimiomètric permet determinar de forma ràpida quins són els factors que més influència tenen en la preparació dels MIPs. Amb aquest mètode s'obre una nova estratègia de síntesi que permet obtenir MIPs amb millor selectivitat de forma ràpida i que pot ser aplicat a la majoria d'analits pel desenvolupament de nous MIPs.

Pel que fa a l'aplicació dels MIPs com a sorbents d'SPE, un dels principals problemes que presenten en alguns casos és la limitada capacitat, la qual esdevé un inconvenient important quan es treballa amb mostres mediambientals on és necessari preconcentrar grans volums de mostra per tal d'assolir els baixos nivells als quals es troben els compostos. Són pocs els estudis [187,205] duts a terme on el MIP hagi demostrat tenir una gran capacitat i per tant que permeti preconcentrar grans volum de mostra (1000 ml) directament a través d'ell, ja que altres treballs on s'han preconcentrat 500 ml d'aigua de riu o de l'aixeta, ho feien primerament a través d'un sorbent comercial [160,188]. Seria interessant doncs, aconseguir sintetitzar MIPs amb elevada capacitat i que per tant permetessin el pas de grans volum de mostra directament a través d'ells.

Una de les aplicacions de la MISPE que encara no està gaire desenvolupada és a l'extracció de compostos en aliments, la qual és també un tipus de mostra complexa on seria interessant aplicar una extracció selectiva amb MIPs. Autors com Cacho [183], Zhang [220] i Zhou [221] han aplicat la MISPE a l'extracció de compostos en mostres tan diverses com vegetals, llevat i blat, respectivament. En tractar-se de mostres sòlides, a l'igual que en el cas del teixit animal o els sediments, després del tractament corresponent a cada tipus de mostra, l'extracte obtingut es passa a través del MIP. Pel que fa a l'estudi de Cacho *et al.* [183] cal destacar el sistema de neteja de la mostra que utilitzen, ja que l'extracte obtingut del pretractament de la mostra es passa primer a través d'un cartutx que conté el polímer de control i l'eluat que s'obté es passa a través del MIP. L'ús del MIP permet discriminar entre la matriu de la mostra i els analits a determinar (triazines), de manera que els analits podien ser extrets quantitativament. En aquest treball, s'assenyala que el fet d'emprar dues etapes en el procés de MISPE no fa menys útil l'aplicació dels MIPs com a materials d'extracció selectius, sinó que ofereix altres possibilitats dins de la seva aplicació en el camp de la química analítica.

La tècnica d'empremta molecular ha estat aplicada a altres dispositius que també permeten realitzar extraccions selectives de compostos. Un exemple són les membranes amb empremta molecular, que maximitzen les interaccions selectives entre l'analit d'interès i la membrana durant l'etapa de filtració de la mostra, normalment aquosa. Hi ha diverses maneres d'introduir reconeixement molecular en una membrana, com per exemple mitjançant una polimerització *in situ* on la síntesi típica del MIP s'utilitza per a la preparació de membranes (polimerització entre l'agent entrecreuant i els monòmers funcionals), o bé introduint les cavitats de reconeixement molecular a materials polimèrics sintètics que inicialment no posseeixen cap reconeixement específic [222,223].

Pel que fa a la preparació de materials inorgànics empremtats, Sellergren *et al.* [224] van dur a terme un estudi on es presentaven uns materials, que normalment actuen com a suport inorgànic (síliques poroses i no poroses), empremtats molecularment i aplicats al camp de la cromatografia de líquids en lloc dels tradicionals polímers d'empremta molecular. Aquest nou dispositiu desenvolupat mostra augment de l'eficàcia i per tant ja no s'obtenen pics amples ni asimètrics com quan s'utilitzen els MIPs convencionals com a columnes cromatogràfiques. Aquesta tècnica pot ser aplicada a una gran varietat de *templates* i la seva utilització en el camp de la SPE i l'electroforesi capil·lar també és possible.

Els polímers amb empremta d'ió metàl·lic (MIIP) [225,226] són semblants als MIPs però presenten reconeixement per ions metàl·lics després dels procés d'empremta, característica aprofitada principalment en aplicacions catalítiques, tot i que aquest tipus de polímer també ha estat aplicat com a fase estacionària en sistemes cromatogràfics, en SPE, en membranes i sensors.

En el darrer *International Workshop on Molecularly Imprinting* (Cardiff, 2004) es va veure com la síntesi de MIPs tendeix a l'obtenció de nanoestructures degut principalment a l'interès que actualment existeix en el desenvolupament d'estructures amb aquestes característiques. En aquest congrés, Haupt *el al.* van presentar un treball on es preparaven superfícies nanoestructurals com a superfícies de reconeixement en sensors òptics.

Com es pot veure, tant la síntesi de MIPs com la seva aplicació sembla que evolucionen en funció de les noves tecnologies. Tot i que aquest materials sintètics han demostrat tenir alguna limitació, els avantatges que ofereixen i la seva versatilitat fa dels MIPs una eina molt potent de gran utilitat i amb molt de futur.

A continuació s'adjunta una còpia del treball enviat a la revista *Trends in Analytical Chemistry* sorgit arran de la revisió bibliogràfica que s'ha fet sobre les principals aplicacions de la MISPE a l'extracció de compostos en mostres ambientals i biològiques. En aquest treball també s'inclouen alguns dels resultats més rellevants de la present Tesi Doctoral, degut a que aquesta revisió s'ha efectuat un cop finalitzada la part experimental de la mateixa.

1.3 *MOLECULARLY IMPRINTED POLYMERS APPLIED TO THE SOLID-PHASE EXTRACTION OF COMPOUNDS FROM ENVIRONMENTAL AND*

MOLECULARLY IMPRINTED POLYMERS APPLIED TO THE SOLID-PHASE EXTRACTION OF COMPOUNDS FROM ENVIRONMENTAL AND BIOLOGICAL SAMPLES

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Abstract

In the present paper we review the current state-of- the art of the use of molecularly imprinted polymers (MIPs) as selective materials in molecularly imprinted solid-phase extraction (MISPE). The application of these synthetic polymers as sorbents allows one to not only to preconcentrate and clean the sample but also to achieve a selective extraction of the target analyte, which is important particularly when the sample is complex and impurities can interfere in the quantification. Thus, in this study we discuss the application of MIPs for the extraction of compounds from complex matrices such as environmental and biological samples.

Keywords: molecularly imprinted polymers; solid-phase extraction; water samples; biological samples

INTRODUCTION

Molecularly imprinted polymers (MIPs) are tailor-made materials with high selectivity for a target molecule. This selectivity arises from the synthetic procedure followed to prepare the MIP in which a template molecule is linked, by covalent bonds or non-covalent forces, to a suitable monomer(s) containing functional groups. This link is responsible for the subsequent specific binding sites imparted into the MIP.

MIPs can be synthesised following three different imprinting approaches [1], the non-covalent, the covalent and the semi-covalent. In all these protocols a template molecule interacts with an appropriate functional monomer to establish specific interactions. Once the polymer is obtained the template is removed from the polymer; conse-quently, the template leaves its imprint in the polymer structure and it is this imprint which is responsible for the recognition properties of the MIP. These imprints or cavities are also known as binding sites.

The selectivity of MIPs has been exploited in several applications [2], such as sensors, capillary electro-chromatography, enantiomeric separations, in solid-phase extractions (SPE) and catalysts.

In the last decade, one of the most promising technical applications based on the use of MIPs has been molecularly imprinted solid-phase extraction (MISPE). Most of the studies performed are focused on the extraction of compounds from biological samples [3-8]. Biofluids have been the samples analysed in most of the biological studies [3-6] with only a few papers having been published reporting the extraction of analytes from tissue samples [7,8].

During the last few years, MISPE has been applied also to the extraction of compounds from other matrices such as environmental samples (water and soils) [9-15], food [16-20], plants [21-24] and tobacco [25] although the number of studies published describing some of these samples types is limited.

The use of MIPs in SPE is advantageous mainly when a selective extraction must be performed and because the commonly used sorbents show a lack of selectivity. Subsequently, MISPE allows the analyte to be not only pre-concentrated but also the other compounds present in the sample matrix to be removed.

In the present paper the current state-of-the art with respect to the application of MIPs as selective materials in SPE is reviewed. We discuss the use of MISPE in relation to the selective extraction of several compounds from water and biological samples. To introduce MIPs and how they can be obtained, a brief description about the different synthetic routes and methods available to prepare and evaluate a MIP is also presented.

MIP SYNTHESIS AND EVALUATION

Synthetic procedure

Three different approaches have been used to prepare MIPs [1]. The non-covalent procedure is the most widely used because it is relatively simple experimentally and the complexation step during the synthesis is achieved by mixing the template with an appropriate functional monomer, or monomers, in a suitable porogen (solvent) [26]. After the polymer synthesis, the template is removed from the resultant polymer simply by washing it with a solvent or a mixture of solvents, and the subsequent rebinding step the template to the MIP exploits non-covalent interactions. All these features offer several advantages over the covalent protocol, in which the formation of covalent bonds between the template and the functional monomer is necessary prior to polymerisation. Furthermore, to remove the template from the polymer matrix after synthesis via covalent protocol it is necessary to cleave the covalent bonds. To this end, the polymer is then refluxed in a Soxhlet extraction or treated with reagents in solution [1,27-29]. The third approach, the semi-covalent approach, is a hybrid of the two previous methods. Thus, covalent bonds are established between the template and the functional monomer before the polymerisation, while once the template has been removed from the polymer matrix, the subsequent re-binding of the analyte to the MIP exploits non-covalent interactions as per the non-covalent imprinting protocol [1].

The binding sites obtained by molecular imprinting show different characteristics depending on the interactions established during the polymerisation. The average affinity of binding site prepared using non-covalent forces bonding is generally weaker than those prepared using covalent methods because electrostatic, hydrogen bonding, π-π and hydrophobic interactions, between the template and the functional monomers, are used exclusively in the formation of the molecular assemblies [1,26]. Moreover, an excess of functional monomer relative to the template is usually required to favour template-functional monomer complex formation and to maintain its integrity during the polymerisation. As a result, a fraction of the functional monomers are randomly incorporated in the polymer matrix resulting in the formation of non-selective binding sites.

On the other hand, when covalent bonds are established between the template and the functional monomer prior to polymerisation, this gives rise to better defined and more homogeneous binding sites than the non-covalent approach since the template-functional monomer interac-tions are far more stable and defined during the imprinting process [1,27-29]. Nevertheless, the non-covalent imprin-ting protocol is still the most widely used method to prepare MIPs because of the advantages that this protocol offers over the covalent approach from a synthetic point of view.

In some polymers prepared by the non-covalent procedure, it has been observed that the binding of the template to the polymer can sometimes be so strong that it is difficult to remove the last traces of template, even after washing the polymer several times [4,30,31]. When the MIP is used, small amounts of residual template can be eluted. This bleeding is a problem mainly when the MIP has to be applied for the extraction of the target analyte at trace levels.

To overcome this drawback, some authors have synthesised MIPs using an analogue of the target molecule as a template (template analogue approach) [31-35]. In this way, if the MIP bleeds template then the elution of the template does not interfere in the quantification of the target analyte. Andersson was the first author to synthesise a MIP using a template analogue [31]. In this case, a MIP selective for sameridine was prepared using as a template a close structural analogue of sameridine. However, it should be pointed out that the use of template analogues is not always the solution, because on one hand, sometimes is not possible to identify and source the suitable analogue and, on the other hand, MISPE is not always used prior a separation technique such as liquid chromatography (LC). For this reason, other methods such as thermal annihilation, microwave-assisted extraction (MAE) and desorption of the template with supercritical fluids have been developed also for removing the template from the MIP [36].

It should also be mentioned that in each polymerisation, a non-imprinted control polymer (NIP) is also synthesised in the same way as the MIP but in absence of the template. The selectivity of the NIP and MIP is compared, which allows the imprinting effect to be evaluated.

It is important to state that MIPs can be obtained in different formats depending on the preparation method followed [37]. To date, the most commonly way to prepare MIPs are by conventional solution polymerisation, suspension polymerisation, precipitation polymerisation, multi-step swelling polymerisation and emulsion core-shell polymerisation. There are also other methods such as aerosol or surface rearrangement of latex particles, but they are not used routinely.

When a MIP is obtained by conventional solution polymerisation, the resultant polymer is a monolith, which has to be crushed before being used, except when the MIP is prepared *in situ*. On the other hand, suspension polymerisation (in fluorocarbons or water) and precipitation polymerisation methods allow MIPs to be prepared in the form of spherical polymer particulates. When multi-step swelling or emulsion core-shell polymerisation methods are followed, then the MIP is obtained as monodisperse seeds. Conventional solution polymerisation is the most commonly used preparation method because of its simplicity and universality. This method does have some drawbacks in that the processes of grinding and sieving are not only wasteful and time-consuming, but they also may produce irregularly sized particles. However, for MISPE applications the lack of regularity in the particle size is not a particular problem.

In the last few years, some improvements concerning the MIP synthesis have been made developing combinatorial libraries, which allow us to check in an easy and fast way the best conditions to obtain the optimal MIP [38]. The development and implementation of these screening techniques is expected to accelerate the discovery of new MIPs.

Evaluation of the selectivity of the MIP

Prior to their use in MISPE experiments, MIPs are usually evaluated to check their recognition properties for a target analyte. Chromatographic evaluation and equilibrium batch rebinding experiments are the methods most commonly used to investigate the selectivity of the imprinted materials [30,39].

Chromatographic evaluation allows one to measure the capacity factors (k') and the imprinting factors (IF) of MIPs [40]. These values are obtained from the retention time of the template molecule on a chromatographic column packed with the MIP and a second column packed with the NIP. If the MIP is imprinted, then the analyte should be retained more strongly on the MIP than on the NIP because of the selective interactions. In some studies [6,14,15,19], the selectivity of the MIP has also been probed using structurally compounds related to the template. If the MIP retains these compounds almost as well or better than the template, then it is an indication that the MIP shows cross-reactivity [14].

For equilibrium batch rebinding experiments, a known mass of template in solution is added to a vial containing a fixed mass of polymer. Once the system has come to equilibration, the concentration of free template in solution is measured and the mass of template adsorbed to the MIP calculated [16,33,35,41,42]. Some of these experiments are based on radio-ligand binding, which represents a very high sensitive method to study the population of binding sites with strongest binding characteristics [41]. Typically, the sample is incubated with the radio-ligand for several hours and a centrifugation step then used to sediment the polymer particles. The radioactivity in the supernatant is then measured.

MISPE APPLICATIONS

MISPE is based on conventional SPE procedures, therefore conditioning, loading, clean-up, and elution steps are performed as a matter of routine. During the conditioning step, the cavities (binding sites) of the MIP are activated in order to maximise the interactions with the target analyte present in the sample.

In the loading step of the MISPE, the sample medium has a direct influence on the recognition properties of the imprinted polymer. Thus, if the sample is percolated through the MIP in a low polarity solvent, a selective loading step can be achieved, in which only the target analyte is selectively retained on the MIP while the sample matrix is non-retained [43]. On the other hand, when the analyte of interest is present in an aqueous medium, the analyte and other interfering compounds are retained non-specifically on the polymer. Consequently, to achieve a selective extraction, a clean-up step with an organic solvent is introduced prior to the elution step [7,9,10]. This clean-up is more critical in MISPE procedures than in conventional SPE.

The clean-up step must be optimised in terms of the pH and nature and volume of the washing solvent in order to exploit the MIP's ability to selectively recognise the target molecule. Thus, the clean-up solvent should suppress the non-specific interactions without disrupting the selective interactions between the MIP and the target molecule. For this purpose, low polarity organic solvents such as dichloromethane, toluene or chloroform are the most widely used [14,15,19]. However, good results have been obtained also with other solvents with high polarity, such as acetonitrile or methanol [3-6,10,12]; nevertheless, some authors state that recognition is often better when the porogen is used as the solvent because the environment established during the synthesis is reproduced (solvent memory effect) [9,44]. Therefore, when water samples are percolated through MIPs, the clean-up step can be problematic because the washing solvent used is usually non-polar, which may give rise to miscibility problems. To avoid this drawback, the MIP can be dried by, for example, drawing air through the polymer [9,10,15,43]. If small amounts of water remain on the cartridge after sample application the binding ability of the MIP may be influenced in the subsequent selective clean-up step. Therefore, a decrease in retention and selectivity may be observed.

Although a clean-up step seems to be essential to achieve a selective extraction, it can be avoided if the elution step is selective enough. In this case, although the analyte and the other components in the sample matrix are retained on the MIP by non-selective interactions during the loading step, a selective elution of the target analyte can be performed using an appropriate solvent. To attain high enrichment factors it is necessary to use small volumes of solvent, but the interactions between the MIP and the analyte are sometimes so strong that the volume of eluting solvent has to be increased. To avoid this, mixtures of organic solvents or an organic solvent with water or with a modifier, such as acetic acid or pyridine, can be used. Water disrupts the specific non-covalent interactions on the one hand, and on the other hand the modifier competes with the binding sites to interact with the target molecule. Both represent an effective manner for rapid elution of the analyte.

When a selective elution is performed, the MISPE can be coupled directly to a specific detection system as has been demonstrated in a study described by Sellergren [45]. In this paper, a MIP imprinted with pentamidine was used to extract pentamidine from human urine. Although pentamidine was the only analyte present in the sample, the clean-up performed by the MIP was so effective that the matrix interferences were completely removed, allowing pentamidine to be directly injected in the detection system (UV detector).

In a similar fashion to SPE using conventional sorbents, MISPE procedures can be performed in off-line and on-line mode coupled to a chromatographic technique, mainly liquid chromatography (HPLC). However, most of the MISPE studies published so far have been developed in off-line mode [3-12,21-23,30-33], not only because of the relatively simple instrumentation required, but also because stronger elution conditions can be used without compromising analytical instrumentation due to solvent or pH or modifier incompatibility.

Off-line MISPE applications

To perform off-line MISPE, polyethylene cartridges are packed with the polymer (typically 15-500 mg). After the conditioning, loading and clean-up (when necessary) steps the extract collected from the elution step is injected into an analytical system, such as liquid chromatography (LC) [3-6,9-12,21-23], gas chromatography (GC) [31] or a capillary electrophoretic system (CE) [47,48].

Off-line MISPE has been applied to the extraction of several compounds from different matrices such as environmental samples (river water, groundwater, waste water from treatment plants, sea water, soil extracts) [9-12,47-51], biofluids (urine, serum, plasma, blood) [3-6,30-33], tissue samples [7,8,52-54], food [15,48,51], and medicinal plants [21-24].

In the last few years, recent developments concerning the extraction of compounds from environmental samples have been described [9-12,15,43,44,47-51]. In Table 1 some of the most relevant studies published so far are shown. The first off-line MISPE paper describing an environmental application was published by Ferrer *et al.* [44]. In this study, a MIP prepared using terbutylazine as the template molecule showed cross-reactivity; subsequently, the MIP was used for the enrichment of six chlorotriazines from ground water and sediment samples. After a clean-up step with 2 ml of dichloromethane, the matrix components that were retained non-specifically on the MIP were removed, and the recovery of all six chlorotriazines was higher than 80% (except for propazine, which was recovered in a 53%) after percolating 100 ml of water sample. In the last five years, several studies focusing on the extraction of triazines from river water have also been published [9,43,48]. Chapius *et al.* [9] and Pap *et al.* [43], followed the same synthetic procedure described by Ferrer *et al.* to prepare a MIP, also imprinted with terbuthylazine, therefore when the polymers were obtained and applied to MISPE they also showed cross-reactivity. Both authors paid special attention to the retention mechanism of triazines on their respective MIPs, concluding that small amounts of water on the MIP (arising from the sample) could decrease considerably the retention of the analyte. For this reason, a drying step was included before the clean-up step. Although Ferrer *et al.* and Chapius *et al.* applied the water sample directly to the MIP and good results were obtained, Pap *et al.* used a coupled system between a C_{18} extraction disk and the MIP cartridge which allowed 500 ml river water to be preconcentrated. Thus, the sample was first passed through the C_{18} disk and the retained analytes eluted with 30 ml of dichloromethane and injected on to the MIP. Therefore, a selective loading step was achieved, in which only the target compounds were retained; subsequently the clean-up step was avoided. To elute the analytes from the MIP, the C_{18} and the imprinted polymer were disconnected. This combined system allowed not only to preconcentrate high sample volumes but also to avoid the presence of water when the sample is percolated through the MIP. In this way, the sample is loaded on the MIP in an organic solvent which enhances the selective interactions with the target analyte.

Table 1. MISPE studies applied to the selective extraction of analytes from environmental samples

Template	MIP synthesis	Sample	MISPE method	Analytical system	Reference
4-nitrophenol	Semi-covalent Non-covalent	River water	On-line	MISPE-HPLC-UV	1 60
Terbutylazine	Non-covalent*	River water	Off-line C_{18}-MIP on-line Off-line On-line	HPLC-DAD Off-line MISPE-HPLC-UV HPLC-DAD RAM-MIP-HPLC-MS	9 43 44 61
1-NS	Non-covalent*	River water	Off-line	HPLC-UV	10
Cyanide ion	Non-covalent metal complex	Waste water	Off-line	HPLC-UV	11
Benzo[a]-	Non-covalent	Tap and	Off-line	HPLC-Fluorescence	12

pyrene		lake water			
p-tert-butylphenol	Non-covalent	River water	Off-line	HPLC-ED	13
Bisphenol A	Non-covalent*	River water	On-line	MISPE-HPLC-fluorescence	62
4-CP	Non-covalent*	River water	On-line	MISPE-HPLC-UV	14
Ametryne	Non-covalent*	Soil	Off-line	HPLC-UV	15
2,4,5-trichlorophenoxyacetic acid	Non-covalent*	River water	Off-line	CE-DAD	47
Propazine	Non-covalent*	Tap water, ground water, soil and corn	Off-line PS-DVB extraction disk and MIP	CE	48
Metsulfuron-methyl	Non-covalent*	Tap water, surface water and rain water	Off-line	HPLC-UV	49
Ibuprofen	Non-covalent*	River water and waste water	Off-line	HPLC-UV	50
Monosulfuron	Non-covalent	Soil	Off-line	HPLC-UV	51
Pirimicarb	Non-covalent	River water	On-line	MISPE-voltametric detector	64

(*) MIPs with cross-reactivity

In contrast, Turiel *et al.* [48] prepared a MIP using propazine as template and acetonitrile or toluene as porogen. The polymerisation was thermal instead of UV initiated as had been the case for the polymer prepared by Ferrer *et al.* [44]. However, the MIP synthesised by Turiel *et al.* showed cross-reactivity also, which enable the MIP to extract a mixture of triazines with recoveries higher than 70% in all instances. In this study, Turiel *et al.* [48] percolated the water sample (500 ml tap water) through two PS-DVB 3M (Empore) extraction disks in the first instance. This allowed the author to elute the retained analytes in an organic solvent, so that the presence of water was avoided during the subsequent retention of the analytes on the MIP as has been previously explained. The analytical technique used was micellar electrokinetic chromatography. Some of the electropherograms obtained in this work are shown in Figure 1, which shows the effect of using a selective material (MIP) in a SPE procedure, since matrix components from tap and ground water samples are nearly completely removed and the analytes selectively retained.

Trends Anal. Chem. (to be p

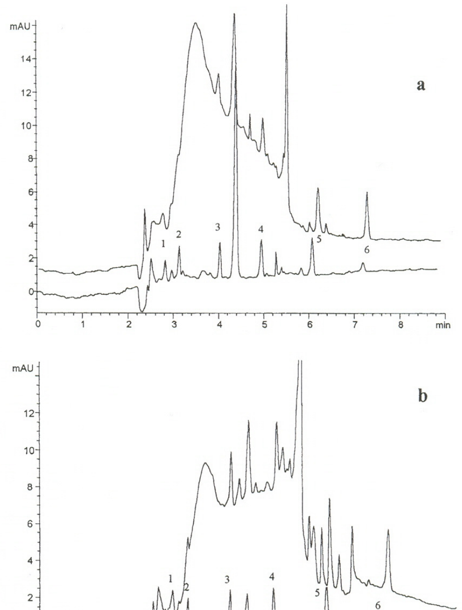

Figure 1. Chromatogram obtained after percolating through the MIP a) tap water extract spiked at 0.1 μg l^{-1} of each triazine with and without clean-up step ; b) ground water extract spiked at 0.5 μg l^{-1} of each triazine with and without clean-up step. Peak assignation: (1) desisoproplatrazine, (2) desethylatrazine, (3) simazine, (4) atrazine, (5) propazine, (6) prometryn [48].

Other environmental pollutants, such as cyanide [11], benzo[a]pyrene [12] and phenoxyacid herbicides [47], have also been used as templates to prepare MIPs in the last few years. Cyanide and benzo[a]pyrene seem more difficult to imprint than most other analytes because cyanide is an ion and a specific metal-complexing polymer has to be prepared, and benzo[a]pyrene is a polycyclic aromatic hydrocarbon (PAH) without any functional group. Nevertheless, both MIPs were successfully applied to the extraction of their respective templates from waste water and surface water.

It is well-known that the concentration of analytes in environmental samples is usually very low, therefore, to target these low levels large volumes of sample have to be percolated through the MIP. This can be a problem because MISPE polymers may suffer from restricted flow-rates and plugging, which can lead to long analysis times. However, in the last few years it has been demonstrated in several studies [10,49,50] that it is possible to percolate large sample volumes of environmental samples such as tap, river and industrial water, directly through the MIP (after a filtration step) with a good flow-rate and an ability to re-utilise the cartridge several times. Zhu *et al.* [49] prepared a MIP using a sulfonylurea herbicide (metsulfuron-methyl) as template, which allowed 1000 ml of tap and river water samples to be extracted. As this MIP also showed cross-reactivity, this feature was exploited to extract a mixture of sulfonylurea herbicides with recoveries not lower than 96%. In some studies described previously by our research group [10,50], it was observed that a MIP imprinted with 1-naphthalene sulfonic acid (1-NS) [10], which showed cross-reactivity, allowed the template molecule and a mixture of eight naphthalene sulfonates (NSs) with different functional groups (hydroxyl (-OH), amino (-NH_2) or nitro (-NO_2)) to be extracted with good recoveries from 500 ml of river water. Good recoveries were also obtained when another MIP synthesised with ibuprofen as template molecule [50], was applied to the selective extraction of a mixture of non-steroidal anti-inflammatory drugs (NSAIDs) from 1000 ml of river water.

In the last few years, some attempts have been made to apply the MISPE procedures to the extraction of environmental pollutants from soil samples, whose extract obtained after an appropriate soil treatment (soxhlet, microwave assisted extraction, etc…), is passed through the MIP. Some of these studies have been previously described [44,48] since the target compound has been extracted also from water samples, but in the studies published by Chapius *et al.* [15] and Dong *et al.* [51] a MISPE procedure has been developed to extract triazines and monosulfuron from soil samples, respectively. Chapius *et al*. developed an extraction method in which the selectivity of the MIP prepared using ametryn as template was compared with an immunosorbent (IS) based on anti-triazines polyclonal antibodies immobilised on silica. It was found that the selectivity of the MIP was comparable to the selectivity of the IS as can be seen in Figure 2.

Off-line MISPE, as applied to the extraction of compounds from biological matrices, is the most widely studied application so far [3-8,31-33,52-55]. Table 2 summarises the extraction conditions of some of the most relevant studies published so far. Several analytes, such as diphenyl phosphate [5], naproxen [6] and amobarbital [32], have been isolated from urine samples; sameridine [31] and querciting [55] from plasma, and other drugs such as propranolol [4] and enrofloxacine [8] have been extracted from several matrices such as urine, plasma, bile and animal tissue. When these analytes have to be extracted from a biofluid, this extract could be applied directly to the MIP after a filtration step. Nevertheless, in nearly all these studies, the biofluid was diluted with an organic solvent to minimise the aqueous content of the sample and to precipitate some proteins. There are only a few studies in which the sample has been applied directly to the MIP [3,6,8,32,55], enabled because the binding of the target analyte on the MIP was complete in aqueous samples, although the synthesis of the polymer was carried out in an organic solvent as usual. In all these studies, the volume of sample was only few millilitres, however, in some research papers reported previously by our research group [6], a MIP prepared with naproxen as the template enabled a mixture of compounds structurally related to the template to be extracted from 25 ml of urine sample. For biological samples, 25 ml can be considered to be a high sample volume; however it is an advantage to use higher volumes when low detection limits have to be achieved. In nearly all these studies, although the sample volume passed through the MIP was not very high, the amount of matrix compounds present made it necessary to apply a clean-up step before eluting the retained analytes. The selectivity arising from the MIP allowed the compound to be quantified without compounds interfering. Some authors have compared MISPE procedures with a conventional SPE and found that the use of a MIP improves considerably the resultant chromatograms [3,6,55].

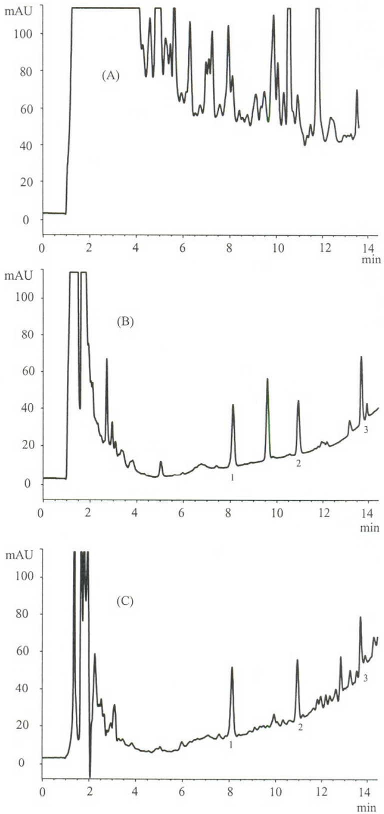

Figure 2. Chromatogram obtained after the injection of a soil extract containing 20 ng g^{-1} of triazines (A) without and (B) with a clean-up step on the MIP and (C) on the anti-triazine immunosorbent. Peak assignation: (1) atrazine, (2) simazine, (3) terbutylazine [15].

Table extraction of analytes from biological samples

-			MISPE method	Analytical system	Reference
p			Off-line	HPLC-MS	5
P			Off-line On-line	HPLC-Fluorescence HPLC-UV	4 59
Naproxen	Non-covalent	Urine	Off-line	HPLC-UV	6
Atrazine	Non-covalent	Beef liver	Off-line	HPLC-UV	7
Enrofloxacin	Non-covalent*	Urine, pig liver	Off-line OASIS-MISPE	HPLC-UV	8
Methyl sameridine	Non-covalent	Plasma	Off-line	GC-FID	31
Amobarbital	Non-covalent	Urine	Off-line	HPLC-photodiode array detector	32
2-aminopyridine	Non-covalent*	Serum	On-line	MISPE-DPE-UV	34
Pentycaine	Non-covalent*	Plasma	On-line	MISPE-LC-MS-MS	35
Verapamil	Non-covalent*	Urine, plasma	On-line	RAM-MIP-LC-MS	42

Pentamidine	Non-covalent	Urine	On-line	MISPE-UV	45
Oxytetracycline	Non-covalent*	Pig kidney	Off-line	HPLC-UV	52
Clenbuterol	Non-covalent*	Bovine liver, urine	Off-line Extrelut-MISPE	HPLC-UV	53
Bromo-clenbuterol	Non-covalent	Bovine liver	MSPD	HPLC-MS	54
Quercitin	Non-covalent	Plasma	Off-line	HPLC-UV	55
Caffeine	Non-covalent*	Urine	On-line	MISPE-HPLC-UV	56
Ibuprofen	Imprinted RAM*	Serum	On-line	HPLC-UV	57
Tramadol	Non-covalent	Plasma	On-line	RAM-MIP-LC-UV	58
Simazine	Non-covalent*	Urine	On-line	C_{18}-MIP-HPLC-UV	63
Cephalexin	Non-covalent	Serum	On-line	MISPE-DPE-UV	67

(*) MIPs with cross-reactivity

Most of the off-line MISPE studies applied to biological samples have been performed using biofluids as the sample. Thus, the number of studies relating to the extraction of compounds from other biomatrices, such as tissue samples [7,8,52-54], is limited. Muldoon *et al.* [7] developed the first study in which a MIP was used to purify tissue sample extracts prior to analysis and quantification. In this paper, a MIP selective for atrazine was synthesised and used as an SPE sorbent to selectively extract this analyte from beef liver samples, as Figure 3 shows.

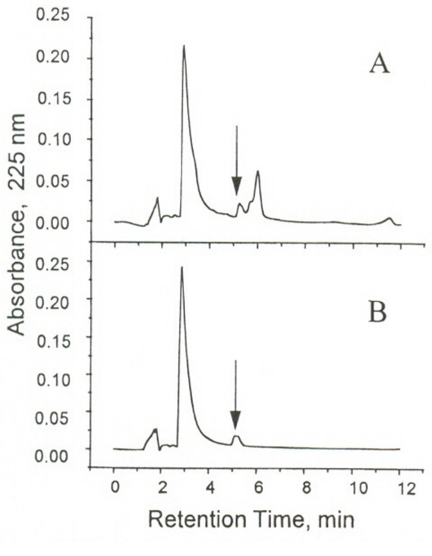

Figure 3. Chromatograms obtained after the percolation of beef liver extract spiked with 0.1 mg l^{-1} of atrazine (a) before MISPE; (b) after MISPE extraction [7].

Tissue is a very complex sample. For this reason, an exhaustive pre-treatment is always required. The tissue is usually homogenised and centrifuged and only the extract is passed through the MIP; however in some cases the matrix is not completely removed. This is a problem because the utility of the MIP and the retention of the target analyte can be reduced since the accessibility to the imprinted sites is hindered by these matrix compounds. For this reason, in some studies [8,53,54] a commercial SPE sorbent is used to clean the sample prior application of the sample to the MIP cartridge. Consequently, the number of impurities decreases and the target analyte can be quantified easily. Moreover, this procedure also allows the analytes retained on the commercial cartridge to be eluted using an organic solvent so that the analytes will then be passed to the MIP in this solvent thus avoiding the presence of water arising from the sample. Brambilla *et al.* [53] used an Extrelut 20 column for a first extraction of clenbuterol from bovine liver and the eluate collected was applied to the MIP. In a previous study developed by our group [8] a MIP prepared with enrofloxacin as template was used to extract enrofloxacin from urine samples and also from pig liver. To this end, the sample was first passed through an OASIS HLB cartridge and the methanolic extract collected passed through the MIP. The use of these two cartridges allowed the analytes to be pre-concentrated in an organic solvent to enhance the imprinted effect of the MIP. Crescenzi *et al.* [54] used a matrix solid-phase dispersion (MSPD) pre-treatment for the extraction of clenbuterol from liver. This procedure, shown in Figure 4, simplifies the pre-treatment of solid samples because the tissue is homogenised and mixed with a commercial SPE sorbent (C_{18}) and an empty cartridge filled with this mixture. However, an additional clean-up step was still required and MSPD was then combined with a MISPE procedure. It should be noted that Crescenzi *et. al.* used bromoclenbuterol as a template analogue in order to avoid bleeding of analyte from the MIP that could interfere in the quantification of the target analyte (clenbuterol).

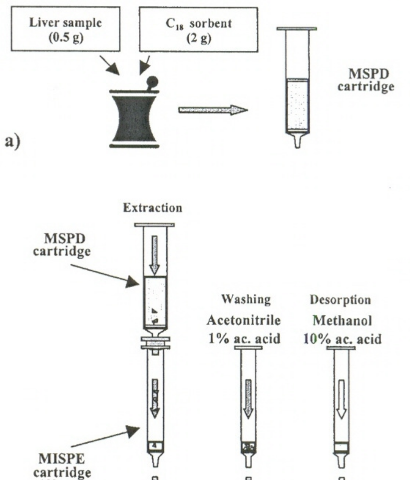

Figure 4. Sample preparation procedure used by Crescenzi *et al.* (a) MSPD cartridge preparation; (b) tandem extraction by MISPE [54].

As has been explained previously, some MIPs show cross-reactivity. Although this feature is exploited mainly in environmental applications, there are also some studies in which this property has been evident in MIPs used in biological applications. Some of these studies have been developed by Brambilla *et al.* [53] in which clenbuterol and other β-adrenergic agents were extracted simultaneously. In our research group, a MIP prepared using enrofloxacin as template [8] allowed enrofloxacin and ciprofloxacin to be extracted from liver samples and to be quantified below the maximum residue limits (MRL) established by the European Union (EU). This cross-reactivity feature is especially important in this case because the MRL is the sum of both compounds.

Trends Anal. Chem. (to be published)

On-line MISPE applications

To automate SPE, on-line MISPE protocols have also been developed. Moreover, on-line systems allow the entire sample passed through the MIP to be directly injected on to the subsequent analytical system.

On-line MISPE studies have been developed mainly by coupling a pre-column packed with a few milligrams of polymer (e.g., 50 mg of the MIP) to a LC system [1,13,14,35,42,56-64] in which the mobile phase of the LC is the eluting solvent of the MISPE process, so that the mobile phase desorbs the analytes retained on the MIP and introduces them on to the analytical column. However, this can be a problem when the mobile phase is not powerful enough to disrupt the specific interactions between the analyte and the MIP. In this scenario, a modifier (such as a strong acid or base) is usually added to the mobile phase in order to disrupt the specific interactions between the MIP and the analyte. However, the use of such modifiers in the mobile phase can be a problem for the chromatographic separation. This is incompatibility is easily overcome when working with off-line systems. Another important distinguishing difference between off-line and on-line MISPE is the drying of the sorbent prior to the clean-up step, which is easier to perform in off-line systems.

Nevertheless, the number of on-line MISPE publications has increased considerably in the last two years [13,14,19,42,49,56-64]. In these studies, the analyte of interest has been extracted from several matrices such as environmental, biological and food samples.

Some of the on-line MISPE studies applied to the extraction of analytes from environmental samples have been developed by our research group [1,14,60]. The study developed by Masqué *et al.* [60] was the first to describe the synthesis and the application of a non-covalently imprinted MIP coupled to a liquid chromatographic system to selectively isolate 4-nitrophenol (4-NP) from a mixture of phenolic compounds in river water samples. In this example the MIP showed very good selectivity for 4-NP but the recovery of this compound decreased after the clean-up step (36%). In order to increase the recovery for this analyte, another MIP was prepared following a semi-covalent approach [1]. In this study, although the recovery of 4-NP was increased (50%) other phenolic compounds were retained slightly on the MIP after applying the clean-up step.

In view of the good results obtained in these particular studies, another MIP design to selectively extract 4-chlorophenol (4-CP) from river water [14] was prepared by following the same protocol described by Masqué *et al.* [60]. In this study, a strong cross-reactivity was observed for all the 4-chlorosubstituted compounds and 4-NP present in the sample after the clean-up step, and the other chloro or nitrophenols were removed selectively during this step as can be seen in Figure 5. Surprisingly, this cross-reactivity was not observed for any of the 4-NP MIPs described previously even though 4-NP and 4-CP are structurally very similar.

Watabe *et al.* [13] prepared a MIP to

selectively enrich bisphenol A (BPA) from water samples (lake and river water). An on-line column-switching HPLC valve allowed BPA to be determined at ultra-low levels (ppt). In a similar fashion, San Vicente *et al.* [62] prepared a MIP selective for BPA. The conditions used to prepare the MIP had been optimised previously in an earlier study [65], in which an experimental design and a multivariate analysis were used for the screening and optimisation of the polymerisation parameters to enhance the selectivity of the MIP and affinity towards the template. In this MISPE study, BPA was selectively extracted from 25 ml of river water fortified with a mixture of several epoxy resins at µg l^{-1} levels.

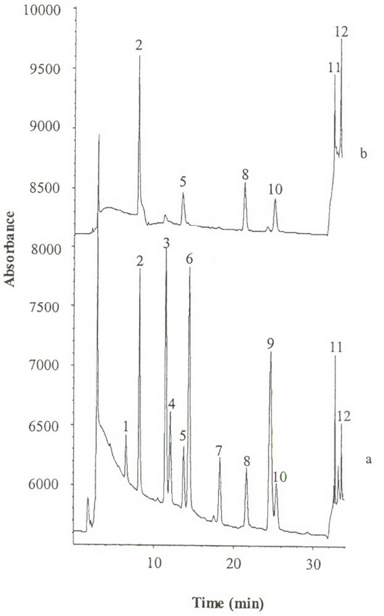

Figure 5. Chromatogram obtained after an on-line sample percolation of 10 ml of river water spiked at 10 µg l^{-1} of each phenolic compound (a) without washing step (b) with washing step using 0.1 ml of dichloromethane. Peak assignation: (1) phenol, (2) 4-nitrophenol, (3) 2,4-dinitrophenol, (4) 2-chlorophenol, (5) 4-chlorophenol, (6) 2-nitrophenol, (7) 2,4-dimethylphenol, (8) 4-chloro-3-methylphenol, (9) 2-methyl-4,6-dinitrophenol, (10) 2,4-dichlorophenol, (11) 2,4,6-trichlorophenol and (12) pentachlorophenol [14].

In the study developed by Mena *et al.* [64] a selective MIP for pirimicarb was on-line coupled to a voltammetric detector. In this work, a clean-up step could be avoided because a selective elution of the target analyte was achieved using a mixture of methanol/water/acetic acid (7:2:1). Pirimicarb was extracted from several waters such as tap water, spring water, river water and sea water, with recoveries for this analyte of between 55-75%, which demonstrates that a flow injection system can be effectively coupled to a MIP microcolumn. Moreover, this methology allows analytes to be pre-concentrated in a rapid (less than 15 min) and simple manner.

In all of the previous on-line MISPE studies, the aqueous sample has been applied directly to the MIP and good imprinting effects observed. However, it has been demonstrated that in some cases, the presence of water can disrupt the specific interactions between the analyte and the MIP. To overcome this drawback, Koeber *et al.* [61] developed an on-line extraction system to extract a mixture of triazine combining two sorbents as has been previously explained for the off-line systems. In this study a restricted access material (RAM) was coupled to the MIP column; the sample was first size-selectively fractionated on the RAM, where the humic substances were efficiently separated. Then, the sample was transferred to the MIP by means of a pure organic mobile phase (acetonitrile). The clean-up step was so efficient that no remaining matrix compounds were detected in the eluate which demonstrated that a selective and efficient sample clean-up was achieved. Mass spectrometry (MS) was then used as a detection system, which offered additional advantages in terms of selectivity and sensitivity.

The first on-line MISPE study applied to the extraction of compounds from biological samples was performed by Sellergren in 1994 [45] as has been described previously. The number of MISPE studies where the MIP is directly coupled to the detection system is still limited and nearly all of them have been developed in a pulsed elution (PE) or differential pulsed elution (DPE) mode [20,34,66,67]. Mullet *et al*. developed this new on-line elution system [66] in which the analyte was retained on the MIP in an organic solvent. Then, after observing the peak from the elution of the solvent, the analyte was eluted rapidly with small volumes of a polar protic solvent and directly injected into the UV detector. Although the subsequent separation system is avoided, to perform the PE it is necessary to use high amounts of solvent to disrupt the non-specific interactions. In this case, a MIP imprinted with theophillyne was used to extract this compound from serum. In spite of the good results, Mullet modified this technique introducing a clean-up step before the elution of the analytes, to completely remove the matrix compounds. This technique known as DPE has been applied to the extraction of ochratoxin A [20] from wheat extracts, 4-aminopyridine from serum [34] and cephalexin [67] from plasma.

Andersson *et al*. [35] also developed an on-line MISPE set-up to extract analgesics from plasma. In this study, in order to avoid the interference of the template molecule due to the bleeding of the MIP in the quantification of the target analyte, a MIP imprinted with pentycaine was used in the extraction a mixture of local anaesthetics, such as bupivacaine and ropivacaine, from plasma. This MIP was coupled to a liquid chromatographic system and MS-MS detection. The analytes were retained on the MIP and, after a washing step using a mixture of water/methanol (80:20) and then acetonitrile; the retained analytes were transferred to a C_{18} analytical column *via* a C_8 stacking column. In a similar way, Theodoridis *et al.* [56] prepared a MIP imprinted with caffeine to selectively extract caffeine from urine, beverages and coffee, and the concentrations of caffeine found in coffee and beverages such as cola were very similar to those reported by the reference method.

Since biological samples are very complex matrices, to prevent plugging of the MIP and subsequently low recoveries, a two step SPE procedure has been developed [42,58,63] in a similar fashion to those off-line MISPE studies with biological samples described previously. The MIP prepared by Mullet *et al.* [42] to extract verapamil from plasma and urine showed cross-reactivity, subsequently the polymer was also used to selectively extract the main metabolites of verapamil. In this study, an MS detector was on-line coupled to a RAM-MIP-LC system to confirm the presence of the analytes in the sample. Bjarnason *et al*. used a C_{18} cartridge instead of a RAM, to extract triazine herbicides from urine samples and apple extracts [63]. In this study, the triazine used as template for the MIP synthesis was simazine, but the MIP also showed cross-reactivity in identical manner to the MIP prepared by Ferrer *et al*. [44]. Using this coupled system, the chromatograms obtained were cleaner than those obtained using only the C_{18} column.

In other studies, a commercial sorbent such as a RAM has been imprinted. This methodology was followed by Haginaka *et al*. [57] and Sanbe *et al*. [59] to imprint a RAM polymer with naproxen and ibuprofen [57] and propranolol [59]. In the study performed by Haginaka, two imprinted materials were prepared, in which naproxen and ibuprofen were used as template molecules. Nevertheless, the naproxen imprinted material showed some bleeding. Subsequently, this polymer could not be used for the naproxen extraction. However, since the ibuprofen imprinted material showed cross-reactivity levels for naproxen, this polymer was then used for the isolation of naproxen from serum samples. It should be noted that the merits of this RAM imprinted material are not only that a selective enrichment can be achieved but also that the pH range of stability is wider compared with non-modified RAM materials [57]. Nevertheless, some authors state that this imprinted material suffers from solvent restriction since the sample should be percolated in an aqueous buffer in order to avoid proteins precipitation [42].

Summary

The advantages that these synthetic materials (MIPs) offer as selective sorbents have been demonstrated. MIPs are easy to obtain and in some studies it has been demonstrated that the selectivity of the MIP is comparable to that from ISs. Moreover, the applicability of MIPs in SPE procedures demonstrates the feasibility of using a MIP in several formats for the extraction of numerous templates from different samples.

However, some features still have to be improved. The bleeding and the low capacity of the MIP are some of the main problems that researchers try to overcome.

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2. PART EXPERIMENTAL I RESULTATS

La millora de les característiques que presenten els sorbents comercials per a l'extracció en fase sòlida ha estat durant els últims anys una via d'investigació en el nostre grup de recerca. Inicialment, els estudis realitzats estaven orientats principalment a l'obtenció de sorbents adients per a l'extracció de compostos polars. Posteriorment, es va proposar el desenvolupament de sorbents selectius per SPE i es van escollir els MIPs per la novetat que suposen aquests materials i pels avantatges que presenten front els ISs. Després d'un estudi inicial amb un MIP i degut als resultats tan satisfactoris que es van obtenir es va continuar i potenciar aquesta via de recerca, en la qual se centra la present Tesi Doctoral.

En aquest capítol s'inclou la part experimental i els resultats obtinguts en els estudis realitzats durant la present Tesi. Aquests resultats han estat publicats o estan pendents de publicació en diferents revistes científiques, de manera que es presenten en forma d'articles. Prèviament a cada estudi s'inclou una breu introducció en la qual s'indiquen els objectius de l'estudi, així com la innovació en el moment de la seva realització.

La part experimental es divideix en cinc apartats, corresponents cadascun d'ells al tipus d'analit emprat com a *template* per a la preparació dels diversos MIPs sintetitzats. Així doncs, en el primer apartat s'inclouen els dos estudis realitzats amb compostos fenòlics (4-nitrofenol i 4-clorofenol) com a *template*. El segon apartat descriu l'estudi d'un MIP sintetitzat amb l'àcid 1-naftalensulfònic. El tercer apartat inclou dos estudis realitzats amb dos antiinflamatoris com a *template*, l'ibuprofen i el naproxen. El següent apartat, el quart, descriu l'aplicació d'un MIP empremtat amb l'antibiòtic oxitetraciclina i el cinquè inclou dos estudis on dues fluoroquinolones, la enrofloxacina i la ciprofloxacina, han estat emprades com a *template.*

Tots aquests MIPs i els corresponents polímers de control (NIPs) emprats en els diferents estudis han estat sintetitzats al *Department of Pure and Applied Chemistry* de la *University of Strathclyde* a Glasgow (UK) sota la tutela del Professor David C. Sherrington i el Dr Peter A.G. Cormack durant dues estades que es van realitzar en aquesta universitat. En tots els estudis que es presenten a continuació, els MIPs han estat preparats seguint el procés d'empremta molecular no covalent, a excepció de l'estudi realitzat amb el 4-nitrofenol com a *template*, on també es va preparar un altre MIP seguint el protocol semi-covalent. Pel que fa a la tècnica de polimerització emprada, en tots els casos va ser en solució convencional ja que permet obtenir MIPs d'una manera relativament senzilla. No obstant, les condicions de síntesi (porogen, monòmer funcional, temperatura etc.) van ser diferents en funció de les característiques de cada *template*.

El tipus de *template* escollit per tal de preparar els diversos MIPs utilitzats en cadascun dels estudis és molt diferent i ha estat escollit per l'interès en la seva determinació i a la vegada per explorar amb compostos de diferents característiques.

L'aplicació dels MIPs com a sorbents en processos d'extracció en fase sòlida (MISPE) és la que ha generat més interès fins al moment i ha estat objectiu de la present tesi. El procés de MISPE a l'igual que la SPE emprant sorbents convencionals pot ser desenvolupada tant en línia com fora de línia amb la posterior tècnica analítica. En els estudis que s'inclouen a continuació l'extracció fora de línia ha estat la més utilitzada i la tècnica analítica ha estat la cromatografia de líquids amb detector ultraviolat-visible (UV-vis).

Les mostres analitzades en els diversos estudis han estat mostres aquoses en alguns casos i biològiques en d'altres. En els primers treballs es va analitzar aigua de riu i degut als bons resultats obtinguts amb aquest tipus de mostra també es va portar a terme l'aplicació de la MISPE a l'extracció de compostos farmacològics (antibiòtics i antiinflamatoris) en mostres biològiques com orina humana i teixits d'animals (ronyó i fetge de porc) per tal d'ampliar el camp d'aplicació.

2.1 POLÍMERS AMB EMPREMTA MOLECULAR PER A L'EXTRACCIÓ SELECTIVA DE COMPOSTOS FENÒLICS MITJANÇANT L'ACOBLAMENT EN LÍNIA DE L'EXTRACCIÓ EN FASE SÒLIDA

El primer estudi que es va realitzar en el nostre grup de recerca per millorar la selectivitat dels processos d'extracció mitjançant un MIP, va ser desenvolupat per Masqué *et al*. [1]. En aquest estudi es va dur a terme la síntesi d'un MIP no covalent i es va aplicar a l'extracció del 4-NP (compost emprat com a *template*) en mostres d'aigua del riu Ebre. Com a resultat d'aquesta aplicació, es va veure que el MIP presentava una gran selectivitat per aquest compost, ja que era capaç d'extreure'l selectivament d'una mescla d'onze compostos nitro- i clorofenòlics. No obstant, la recuperació pel 4-NP no era molt elevada i per això es va plantejar la síntesi d'un altre polímer empremtat amb el mateix *template* però modificant algunes de les condicions de síntesi.

D'aquesta proposta sorgeix el primer estudi de la present Tesi Doctoral. Per tal d'intentar millorar els resultats obtinguts en el primer treball de Masqué *et al*. [1] pel que fa a les recuperacions obtingudes, es van preparar dos nous MIPs, on les condicions de síntesi emprades són les que es descriuen a la Taula 2.1.

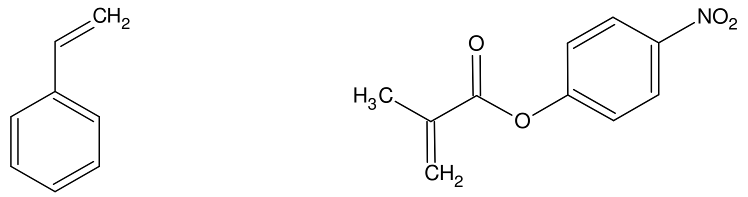
Taula 2.1. Condicions de síntesi pels diversos MIPs empremtats amb 4-NP.

Polímer	Protocol de síntesi	Template	Monòmer
Masqué [1]	No covalent	4-NP	4-VP
P1	No covalent	4-NP	MAA
P2	Semi-covalent	4-nitrofenil metacrilat	Estirè

Porogen: ACN Entrecreuant: EGDMA Iniciador: AIBN MIP: monòlit

Com es pot observar en aquesta taula, P1 només difereix del polímer preparat per Masqué en el monòmer funcional emprat. En aquest cas el 4-NP estableix enllaços d'hidrogen amb el monòmer funcional (MAA), a diferència del MIP preparat per Masqué *et al*. on el MIP a més a més de les interaccions d'enllaç d'hidrogen podia formar també interaccions tipus π-π entre l'anell aromàtic del 4-NP i del monòmer funcional (4-VP).

En canvi, per la síntesi de P2 es va modificar el protocol de preparació (semi-covalent) el qual requereix partir inicialment d'un complex *template*-monòmer com a molècula *template*. En aquest cas el complex emprat va ser el 4-nitrofenil metacrilat, l'estructura del qual es mostra a la Figura 2.1. En aquesta figura també es pot veure l'estructura del monòmer funcional addicional (estirè) que es va afegir per tal de mantenir constant la relació entre el template: monòmer funcional: entrecreuant (T:M:X) emprada en les síntesis anteriors.

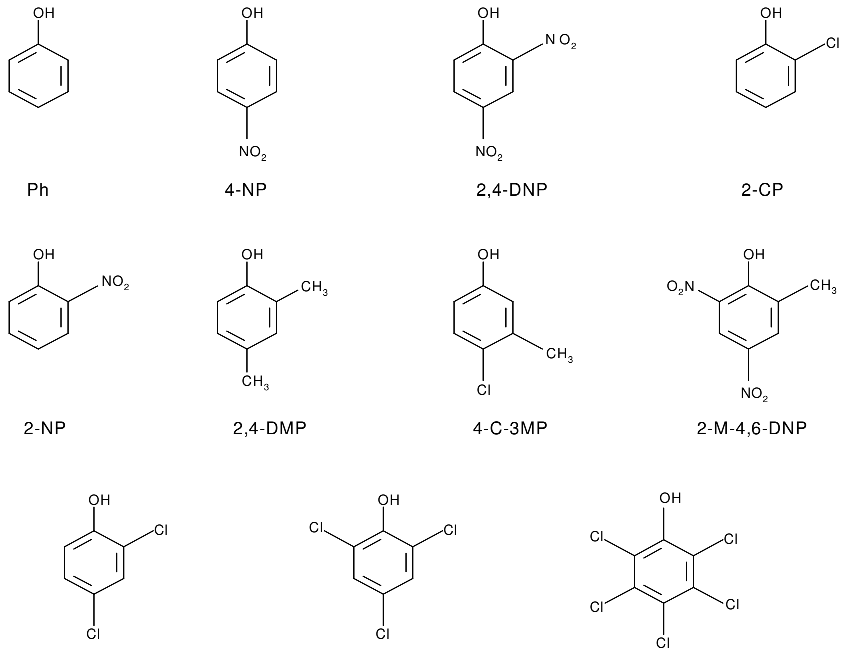


Monòmer funcional adicional estirè

Complex template-monòmer funcional 4-nitrofenil metacrilat

Figura 2.1 Estructura dels reactius emprats com a template i monòmer funcional per a la síntesi del MIP semi-covalent.

P1 i P2 es van aplicar primerament a l'extracció d'una mescla de compostos fenòlics (Figura 2.2) en aigua Milli-Q. El sistema d'extracció emprat es va acoblar en línia a la cromatografia de líquids amb columna de fase invertida i detecció ultraviolada. Per tal d'evitar l'eixamplament dels pics cromatogràfics degut a aquest acoblament, els analits retinguts al MIP es van eluir amb el solvent orgànic de la fase mòbil (ACN/àcid acètic (99:1)) i en sentit contrari al de preconcentració [2]. L'acoblament de la MISPE a la posterior tècnica analítica no és molt freqüent ja que normalment es treballa emprant sistemes fora de línia. En aquest cas però, va ser possible desenvolupar un sistema en línia degut a que el solvent d'elució òptim dels analits retinguts al MIP era perfectament compatible amb el subseqüent sistema analític.



Ph 4-NP 2,4-DNP 2-CP

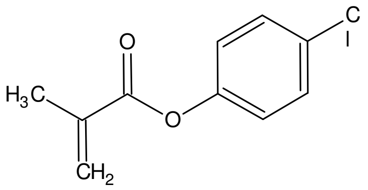
2-NP 2,4-DMP 4-C-3MP 2-M-4,6-DNP

2,4-DCP 2,4,6-TCP PCP

Figura 2.2 Estructura dels compostos nitro- i cloro- fenòlics presents amb el 4-NP duran l'extracció d'aquest

Un cop optimitzat el procés de MISPE, tots dos MIPs van ser aplicats a l'extracció d'aquests compostos en aigua del riu Ebre per avaluar tant la selectivitat com les recuperacions obtingudes quan els polímers eren aplicats en aquest tipus de matriu. Tot i que la selectivitat mostrada pels MIPs reduïa en gran part la càrrega orgànica, principalment àcids húmics i fúlvics que presenten aquestes mostres, es va afegir sulfit sòdic a la mostra [3] per acabar d'eliminar-la i així quantificar correctament el 4-NP. Una vegada establertes les condicions per a l'anàlisi de mostres reals, es van determinar diversos paràmetres analítics com la linealitat i la repetibilitat.

Emprant com a *template* un altre compost fenòlic es va realitzar un segon estudi. En aquest cas el *template* emprat va ser el 4-CP i la síntesi d'aquest polímer també es va dur a terme mitjançant dos protocols diferents, el no covalent i el semi-covalent. Seguint el protocol no covalent, es van preparar dos MIPs, un amb la 4-VP (P1) com a monòmer funcional i un altre amb el MAA (P2). Mitjançant el protocol semi-covalent es va obtenir el tercer MIP pel qual va ser necessari partir del complex 4-clorofenil metacrilat (Figura 2.3) com a *template* i estirè com a monòmer funcional addicional pels mateixos motius que en el treball anterior.



Complex *template*-monòmer funcional
4-clorofenil metacrilat

Figura 2.3 Estructura dels reactius emprats com a template i monòmer funcional per a la síntesi del MIP semi-covalent

El sistema analític emprat va ser el mateix sistema en línia que el descrit en l'estudi anterior i els compostos estudiats també. Després del procés d'optimització de la MISPE, es va veure com els polímers P2 i P3 d'aquest estudi no mostraven un clar efecte d'empremta molecular. Sorprenentment, el que es va observar en el cas de P1 va ser una marcada reactivitat creuada pel 4-NP i per a tots els compostos que presentaven un àtom de clor a la posició 4 de la seva estructura, fet que no havia estat observat prèviament per a cap dels MIPs empremtats amb el 4-NP.

El polímer P1 va ser aplicat a l'extracció de la mescla de compostos fenòlics en aigua del riu Ebre. En aquest estudi, l'etapa de neteja amb el solvent orgànic va ser suficient per a eliminar completament la banda inicial del cromatograma corresponent als húmics i fúlvics i per tant no va ser necessària l'addició de sulfit a la mostra. La linealitat i repetibilitat també es van determinar per a aquest MIP.

Els dos treballs que s'adjunten a continuació inclouen els resultats obtinguts en aquests estudis. En el primer treball (apartat 2.1.1) s'inclou la síntesi i aplicació del MIP empremtat amb el 4-NP, i ha estat publicat en la revista *Journal of Chromatography A 963 (2002) 169*; la síntesi i aplicació corresponent al MIP empremtat amb el 4-CP s'inclou en un segon treball (apartat 2.1.2) i ha estat publicat a la revista *Journal of Chromatography A 995 (2003) 233.*

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2.1.1 *Non-covalent and semi-covalent molecularly imprinted polymers for selective on-line solid-phase extraction of 4-nitrophenol from water samples*

NON-COVALENT AND SEMI-COVALENT MOLECULARLY IMPRINTED POLYMERS FOR SELECTIVE ON-LINE SOLID-PHASE EXTRACTION OF 4-NITROPHENOL FROM WATER SAMPLES

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Abstract

Two molecularly imprinted polymers (MIPs) have been synthesised for the selective extraction of 4-nitrophenol (4-NP) from water samples. One polymer was synthesised via a non-covalent approach and the other via a semi-covalent approach. The selectivity of the polymers for 4-NP was evaluated when these polymers were applied in on-line solid-phase extraction (MISPE) coupled to reversed-phase HPLC. The MISPE conditions for both MIPs were optimised and a clean-up step was included to eliminate non-specific interactions. Differences between the two MIPs were observed with the non-covalent MIP being the more selective of the two, whereas the recoveries were slightly higher for the semi-covalent MIP. The performance of the imprinted polymers in the MISPE of real water samples was also evaluated.

Keywords: 4-nitrophenol; On-line solid-phase extraction; Semi-covalent molecularly imprinted polymer; Water sample

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INTRODUCTION

Nowadays, one of the most interesting objectives for analytical chemistry researchers is to improve the selectivity of the sorbents used in solid-phase extraction (SPE), since many current SPE materials retain not only the target analytes but also other matrix components. This objective is parti-cularly important when analysing complex matrices such as waste water or river water samples, whose humic acids may interfere in the determination of the analytes of interest. In the context of selective sorbents, two types are particularly important [1], the immunosorbents, which have been applied to various types of matrices, and the recently introduced molecularly imprinted polymers (MIPs), whose application in SPE is now being actively researched [2].

The selectivity of MIPs arises because the target analyte (template) is present in the polymerisation mixture during synthesis of the MIP. Once the highly crosslinked polymer has formed, the template molecules are removed from the polymer matrix revealing selective binding sites in the polymer matrix. As a consequence of these binding sites (i.e. molecular recognition sites), the molecularly imprinted polymer is able to selectively recognize the template molecule from other components in a complex sample [3].

Currently, a number of distinct approaches have been used to prepare molecularly imprinted materials. One of these is the pre-organized approach (covalent imprinting), which involves the formation of covalent bonds between the functional monomers and the template molecules prior to polymerisation. Thus the template molecules need to be chemically modified with the functional monomers, and after polymerisation the template molecule is removed from the imprinted polymer by cleavage of the covalent bonds via which it is attached to the polymer. Upon rebinding of the analyte (template) to the polymer the covalent bonds are reformed. Another methodology is the self-assembly approach (non-covalent imprinting) where relatively weak non-covalent intermolecular interactions, such as electrostatic interactions, hydrogen bonding, π-π bonding and hydrophobic interactions, between the template and the functional monomers serve to form molecular assemblies. Hence the selection of functional monomers which interact strongly with the template is crucial to generate high affinity binding sites [4-6].

A third approach is the semi-covalent approach in which the template is covalently bound to a functional monomer during polymerisation, as in the covalent approach, whereas only non-covalent interactions are exploited during the rebinding [7,8]. The fact that the template is covalently bound to the functional monomer at the outset, can in principle yield imprinted polymers with higher binding capacities since there is much better binding site integrity during polymerisation.

It is generally believed that covalent imprinting gives better defined and more homogeneous binding sites than the non-covalent approach since the template-functional monomer interac-tions are far more stable and defined during the imprinting process than the template-functional monomer complex in the non-covalent approach. However, the general applicability of the pre-organized approach is limited because it can be difficult to design suitable binding sites for the target molecule in which covalent bond formation and cleavage are readily reversible under mild conditions. In contrast, non-covalent imprinting is much more flexible in terms of the binding sites that can be exploited and therefore the range of templates which can be targeted. Furthermore, the non-covalent approach is experimentally simpler to realize than covalent imprinting methods because the complexation step is achieved simply by mixing the template with the functional mono-mer(s) in a suitable solvent. No che-mical derivatisation of the template is required, and template removal typically involves simply washing the polymer repeatedly with a suitable solvent or solvent mixture. A major drawback of non-covalent systems is the una-voidable heterogeneity of the binding sites obtained arising from the multitude of complexes formed between the template and the functional monomers which are apparently preserved to some extent during the polymerisation. The non-covalent bonding is generally not strong and thus an excess of functional monomer relative to the template is usually required to favor template-functional monomer complex formation and to maintain its integrity during the polymerisation. As a result, a fraction of the functional monomers are randomly incorporated in the polymer matrix resulting in the formation of non-selective binding sites [7-11].

The potential range of applications for MIPs is very extensive [12,13]. Although the application of MIPs as sorbents in molecularly imprinted SPE (MISPE) was firstly described in 1994 [14], few works have been developed [15]. MISPE has been mainly applied in off-line mode to chromatographic systems, with few applications having been developed thus far in on-line mode [16-19]. In two of these on-line methods [17,18], two successive pre-columns packed with C_{18}-silica and MIP sorbents respectively were on-line coupled to a liquid chromatographic system to extract selectively a group of triazines from environmental water. On the first pre-column, which contained the C_{18}-silica, all the compounds were retained. When they were eluted subsequently from this pre-column only the template and the related structural compounds were retained by the second pre-column which contained the MIP. In a similar way, Koeber et al. [20] also used two successive pre-columns, but in this case the first one packed with a restricted access material (RAM) and the second one with a MIP to selectively extract triazines from water river samples. In contrast, in an on-line MISPE application developed by our group [16] only one pre-column, containing a MIP, was required for the selective extraction of 4-nitrophenol from environmental water. Haginaka and Sanbe [19] also used one pre-column, a combined RAM-MIP pre-column, to extract ibuprofen from plasma. The use of only one pre-column as opposed to two in on-line MISPE clearly offers significant advantages in terms of the ease of method development.

Most MISPE research has been performed with biological samples [18,19,21-23] with the use of MIPs for the analysis of complex matrices of environmental origin being in its infancy. There are a few such applications based on the determination of pesticides in water samples [17,18,20,24-26]. As mentioned above, in a recent paper from our group [16], a MIP for 4-nitrophenol (4-NP) was synthesised and evaluated for on-line MISPE. This non-covalently imprinted polymer used 4-vinylpyridine as the functional monomer and it enabled the selective extraction of 4-NP from river water samples even when other phenolic compounds were present. Joshi et al [7] also synthesised a MIP for phenolic compounds but, in this case, it was a semi-covalent MIP useful in separating phenol from anisole.

In this paper, a detailed study is presented in which the performance of a non-covalently imprinted 4-NP polymer is compared with the performance of a semi-covalently imprinted 4-NP polymer in the on-line MISPE of 4-NP from environmental water. Both polymers exploit an identical, methacrylic acid-based, binding site. To our knowledge this is the first MISPE application of a semi-covalently imprinted sorbent.

EXPERIMENTAL

Reagents and standards

The chemicals for the polymer synthesis were 4-NP, methacrylic acid (MAA) and ethylene glycol dime-thacrylate (EGDMA) from Aldrich (Steinheim, Germany), styrene from Fisher Chemicals (Loughborough, U.K.), 2,2'-azobisiso-butyronitrile (AIBN) from Acros Organics (Geel, Belgium) and acetonitrile from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use via standard procedures in order to remove stabilisers. The AIBN was recrystallised from acetone and the acetonitrile dried over molecular sieves. The monomer-derivatised template, 4-nitrophenyl methacrylate, was synthesised accor-ding to a protocol described in literature [27].

The HPLC-grade solvents were sourced from either Rathburn Chemicals or SDS (Peypin, France) and the water collec-ted from a Millipore water purification system (Milli-Q water). The acetic and hydrochloric acids were from Probus (Badalona, Spain) and dichloromethane from SDS (Peypin, France). The structurally related phenolic pollutants used to investigate the selectivity of the polymers were the 11 priority US Environmental Protection Agency (EPA) phenolic compounds: phenol (Ph), 4-NP, 2,4-dinitrophenol (2,4-DNP), 2-chlorophenol (2-CP), 2-nitrophenol (2-NP) 2,4-dimethylphenol (2,4-DMP), 4-chloro-3-methylphenol (4-C-3-MP), 2-methyl-4,6-dinitrophenol (2-M-4,6-DNP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP), and were all supplied by Aldrich, except for PCP which was from Jansen Chemie (Geel, Belgium).

Instrumentation

In the MISPE study a Must column-switching device (Spark Holland, Emmen, Netherlands), a Waters (Milford, MA, USA) M45 pump and 10 x 3 mm i.d. stainless steel pre-columns, laboratory-packed with ~ 40 mg of the in-house synthesised polymers, were used. These pre-columns were on-line coupled to a liquid chromatographic system which consisted of two LC-10AD pumps, a DGU-4A degasser, a CTO-10A oven and a SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). Having two pumps enables the compounds retained on the pre-column to be eluted only by the organic solvent of the mobile phase. Upon elution, the organic solvent is mixed with the aqueous solvent to form the mobile phase that separates the analytes on the analytical column. The loop for direct injection was 20 µl and the analytical column was a 25 x 0.4 cm i.d. Spherisorb ODS2, 5 µm, supplied by Teknokroma (Barcelona, Spain).

Synthesis of the Imprinted Polymers

Polymer P1 was prepared by the non-covalent approach with 4-NP as the template molecule and MAA as the functional monomer. The pre-polymerisation mixture comprised 4-NP (2.15 mmol), MAA (8.58 mmol), the cross-linking monomer EGDMA (42.90 mmol) and the initiator AIBN (0.90 mmol) dissolved in the porogen acetonitrile (11 ml) in a 25 ml thick-walled glass tube.

A reference, non-imprinted polymer, B1, which did not contain any template, was prepared simultaneously using the same protocol as for P1.

Polymer P2 was prepared by the semi-covalent approach. The pre-polymerisation mixture comprised 4-nitrophenyl methacrylate (2 mmol), styrene (6 mmol), the crosslinker EGDMA (40 mmol) and the initiator AIBN (0.88 mmol) dissolved in the porogen acetonitrile (10.5 ml) in a 25 ml thick-walled glass tube. An additional functional monomer (styrene) in order to keep the template/functional monomer/crosslinker ratio nominally the same for the semi-covalent MIP as for the non-covalent MIP. Styrene was chosen because this gave the opportunity of potentially exploiting π-π interactions in addition to the covalent interaction during the imprinting step.

All three polymerisation mixtures were cooled on an ice bath, sparged with oxygen-free nitrogen for five minutes, sealed under nitrogen and then left to polymerise in a water bath at 60 ºC for 20 h. P1 and B1 polymer monoliths were crushed, ground and wet-sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 μm suitable for the MISPE evaluations. The dry, crushed and ground, polymer P2 was refluxed initially with aqueous 2 M NaOH for 6 h in order to free it from template by breaking the covalent bonds linking the template to the polymer. The resultant polymer suspension was cooled and filtered under vacuum, and the polymer then washed successively with 0.1 M HCl (until the pH of the filtrate was <7), 200 ml of water and 200 ml of methanol. Finally it was dried under vacuum and sieved to obtain regularly sized particles with diameters between 25 and 38 μm. Elemental microanalysis showed that there was no nitrogen present after NaOH treatment, which demonstrated that the template had been successfully removed.

Chromatographic Conditions

The mobile phase consisted of Milli-Q quality water, acidified to pH 2.5 with acetic acid, as solvent A and acetonitrile (containing 1% (*v/v*) acetic acid) as solvent B. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was 15-25% B from 0-10 min, 30% B at 25 min, 100% B at 34 min and then isocratic elution for 2 min. Afterwards, the mobile phase was returned to its initial composition over 2 min. The post-run time was 10 min. The oven temperature was set at 65 ºC and all compounds were detected at 280 nm, except for PCP which was detected at 302 nm.

On-Line MISPE Procedure

For on-line MISPE the polymers were conditioned with 2 ml acetonitrile and 2 ml acidified Milli-Q water (pH 2.5). The spiked water sample (adjusted to pH 2.5) was applied to the conditioned pre-column, and the polymer then washed with 0.2 ml (P1) or 0.5 ml (P2) of dichloromethane and 2 ml Milli-Q water (pH 2.5). Flow-rate was 2 ml min^{-1} in all these steps. The retained analytes were desorbed using solvent B alone and in the back-flush mode to reduce band-broadening, then transferred on-line to the analytical column. Both solvent A and solvent B were mixed prior to reaching the analytical column (Figure 1).

When real samples were used they were filtered through a 0.45 µm filter and adjusted to pH 2.5 before MISPE.

RESULTS AND DISCUSSION

Three different polymers (B1, P1 and P2) were synthesised using methacrylic acid as a functional monomer. B1 (blank) was synthesised in the absence of template, P1 was synthesised via a non-covalent approach and P2 via a semi-covalent approach. All three poly-mers were evaluated subsequently via on-line MISPE.

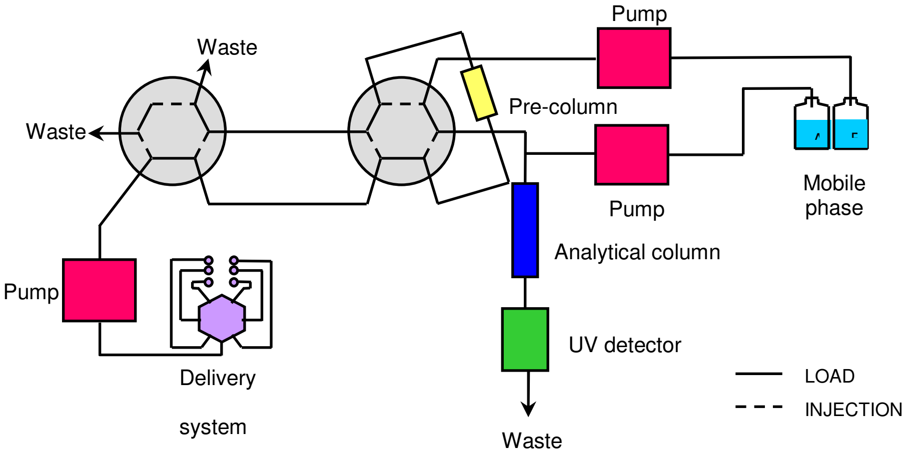

Figure 1. Set-up of the system used.

On-line MISPE

To evaluate the polymers via on-line MISPE they were packed into stainless-steel pre-columns, and before use they were washed with solvent B (acetonitrile containing 1%(*v*/*v*) acetic acid) to verify that there was no residual template (4-NP) present. To confirm that the polymers were imprin-ted and to investigate the selectivity of polymers for 4-NP when this phenol was present with the other 10 priority EPA phenolic compounds in a water sample, an extraction step was develo-ped. Initially, 10 ml of spiked (10 µg l^{-1} of each analyte) Milli-Q water, previously adjusted with HCl to pH 2.5, was passed through the sorbent. All compounds, except for PCP, were retained on the MIPs (Figures 2a and 3a) when a clean-up step was not carried out.

This result can be explained by the fact that under such aqueous loading conditions the analytes interact with the sorbent primarily by hydrophobic interactions (non-specific interactions) which arise between all the analytes and the MIP. To increase the selectivity of the extraction, it was necessary to include a clean-up step with an organic solvent. In such a clean-up step the templated analyte (4-NP) remains strongly bound to the polymer in the imprinted sites whereas the non-templated analytes, which are non-selectively and therefore relatively weakly bound, are washed straight off the MIP. Dichloromethane was selected as the organic solvent because good results were obtained when applying this solvent in previous work [16]. In the case of the blank polymer, a clean-up step with 0.2 ml of dichloromethane stripped all the phenols, including 4-NP, from the pre-column, which indicated that there were no selective binding sites in the blank, as expected.

When the non-covalent MIP, P1, was studied, different volumes of dichloromethane were tested (0.1, 0.2 and 0.3 ml). Figure 2 shows that when 0.1 ml of this organic solvent was applied in the clean-up step not all of the non-selectively bound analytes had been washed off the pre-column. However, when the volume of the washing solvent was raised (0.2 and 0.3 ml), the imprinting effect was clearly evident, since only 4-NP was retained by the pre-column whereas the rest of phenolic compounds were eluted by the dichloromethane. Therefore, 0.2 ml of dichloromethane was chosen as the optimum volume of washing solvent because with this volume the retention of 4-NP was already selective. These results are shown in Table 1. Here it can be seen that the recoveries for 2,4-DNP and 2-CP are not included because they co-eluted and thus their recoveries could not be calculated. PCP is not included since it was not retained by the pre-column in the loading step prior to the clean-up step.

With the semi-covalent polymer, P2, when no clean-up step was used the recoveries were slightly higher than for the non-covalent MIP, presumably due to the higher hydrophobicity of styrene-containing P2.

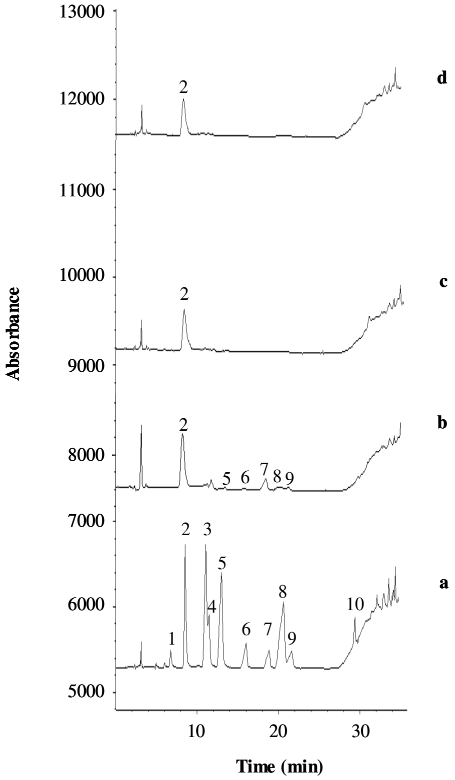

Figure 2. Chromatograms obtained by on-line MISPE with the non-covalent 4-NP imprinted polymer (P1) of 10 ml standard solution (pH 2.5) spiked at 10 µg/l with each phenolic compound. (a) Without washing step, and (b, c, d) With washing step using 0.1, 0.2 and 0.3 ml of dichloromethane, respectively: (1) Ph, (2) 4-NP, (3) 2,4-DNP, (4) 2-CP, (5) 2-NP, (6) 2,4-DMP, (7) 4-C-3-MP, (8) 2-M-4,6-DNP, (9) 2,4-DCP, (10) 2,4,6-TCP

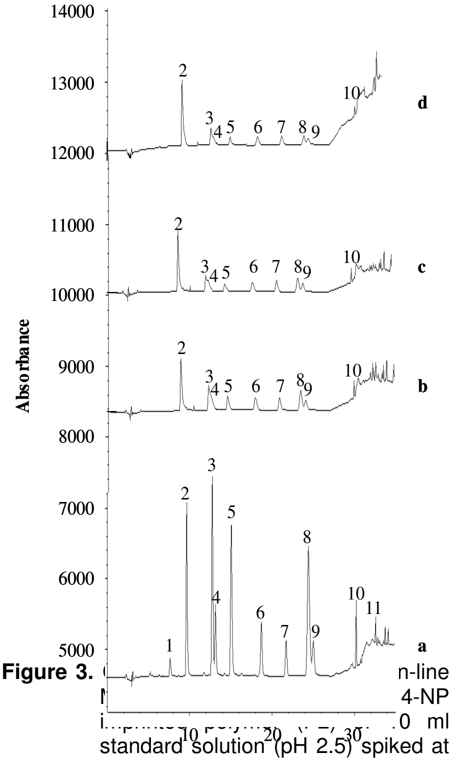

Figure 3. n-line 4-NP .0 ml standard solution (pH 2.5) spiked at 10 µg/l with each phenolic compound. Without washing step (a), and with washing step using 0.2 (b), 0.3 (c) and 0.5 ml of dichloromethane (d). Peak designation, as per Figure 2.

When 0.2 ml of dichloromethane was used in the clean-up step the recovery of some of the phenolic compounds was still high, thus a larger volume of organic solvent was tested (0.3 and 0.5 ml). The results are shown in Table 2. Figure 3 shows the effect of changing the volume of dichloro-methane and it can be seen that even when 0.5 ml of this solvent was used some phenolic compounds were still retained. However, the recovery of 4-NP is similar for the different clean-up volumes tested, which can be explained because with 0.2 ml of washing solvent presumably all the non-specific interactions have already been eliminated. From the results obtained, a volume of 0.5 ml was selected as the optimum. Even larger volumes of this organic solvent were not tested because as the volume of the washing solvent was increased, the recovery of the other phenolic compounds decreased slowly.

If we compare the results obtained for the non-covalently imprinted polymer with those of the semi-covalently imprinted polymer, an important difference is seen between them in terms of the selectivity that they show for 4-NP.

The non-covalent MIP (P1) is more selective than the semi-covalent MIP (P2) since with only 0.2 ml of dichloromethane all the analytes, except for 4-NP, were eluted from the P1 pre-column. In contrast, when P2 was used some analytes remained on the polymer even when 0.5 ml of dichloromethane was used. However, the recovery of 4-NP stayed constant for P2 even as the volume of the washing solvent was increased.

Table 1. Recoveries (%) obtained by washing the non-covalent 4-NP imprinted polymer (P1) with different volumes of dichloromethane following the pre-concentration of 10 ml of a standard solution spiked at 10 µg/l for each analyte[a]

Analyte	Volume of CH_2Cl_2 (ml)			
	0	0.1	0.2	0.3
Ph	38	-	-	-
4-NP	69	68	52	46
2-NP	66	3	-	-
2,4-DMP	54	6	-	-
4-C-3-MP	58	48	-	-
2-M-4,6-DNP	56	6	-	-
2,4-DCP	46	18	-	-
2,4,6-TCP	38	-	-	-

[a] RSDs were lower than 10% in all instances (n= 3)

Table 2. Recoveries (%) obtained by washing the semi-covalent 4-NP imprinted polymer (P2) with different volumes of dichloromethane following the pre-concentration of 10 ml of a standard solution spiked at 10 µg/l for each analyte[a]

Analyte	Volume CH_2Cl_2 (ml)			
	0	0.2	0.3	0.5
Ph	38	-	-	-
4-NP	78	52	51	50
2-NP	71	7	3	2
2,4-DMP	75	20	13	5
4-C-3-MP	68	30	25	12
2-M-4,6-DNP	67	11	7	5
2,4-DCP	62	21	16	9
2,4,6-TCP	56	15	7	4

[a] RSDs were lower than 10% in all instances (n= 3)

The fact that the recovery of 4-NP did not decrease for the semi-covalent polymer may be attributed to the higher capacity of this polymer, derived from the fact that the template was covalently bound to the monomer during polymerisation, consequently with better binding site integrity as a result. Lower selectivity may be due to the fact that many of the binding sites offer only one point of attachment to the analyte, compounded by the fact that sacrificial spacer approach was not employed.

The effect of the sample volume on the recovery was tested by passing different sample volumes through the pre-column (10, 20 and 50 ml). The concentration of analytes was different but the mass of each analyte was constant (0.1 µg). When P1 was tested, and the clean-up step was carried out with 0.2 ml of dichloro-methane, the recovery decreased to 52, 40 and 20% respectively for each of the sample volumes (%RSD (n=3) lower than 12% in all cases). From these results, a volume of 10 ml was selected as the optimum value for further experiments. For P2, when 0.5 ml of dichloromethane was used, the recovery values were 50, 48 and 22% when 10, 20 and 50 ml sample volumes, respectively, were pre-concentrated. Thus, a volume of 20 ml was selected for further experiments because recovery was similar to that with the 10 ml sample and higher sample volumes involve lower detection limits.

If one compares the results obtained for the two MIPs described in this paper with the results obtained for the previously reported non-covalently im-printed MIP [16] prepared with 4-vinylpyridine as the functional monomer and 4-NP as the template, it can be concluded that P1 and the non-covalent

4-vinylpyridine MIP [16] show similar recovery values for all the compounds for the same sample volume (10 ml) when the clean-up step was omitted. However, when the clean-up step is included the recovery of 4-NP is lower for P1 under all conditions. The higher retention of 4-NP on the 4-vinylpyridine based polymer can be attributed to ionic interactions between 4-NP (acidic) and 4-vinylpyridine (basic).

If P2 and the non-covalent MIP using 4-vinylpyridine as the functional monomer [16] are compared, it can be concluded that the recovery values for most compounds are higher in the case of P2 when the clean-up step is omitted. However, when the washing step is included in the comparison, the re-covery for 4-NP is slightly lower for the semi-covalent MIP than for the non-covalent MIP. In spite of this, when the volume of the organic wash solvent is increased with the non-covalent MIP (0.4 and 0.6 ml), the recovery for 4-NP slightly decreases. So it appears that the non-specific interactions between 4-NP and the MIP are not totally eliminated when 0.4 ml of dichlorome-thane was used in the clean-up step. In contrast, the recovery of 4-NP is constant even though the volume of dichloromethane is varied from 0.2 to 0.5 ml when the semi-covalent polymer is used. This implies that the non-se-lective interactions between 4-NP and the polymer are totally eliminated with 0.2 ml of wash solvent.

MISPE of Real Water Samples

To evaluate the performance of the MIPs in the extraction of 4-NP from real samples, Ebro river water was chosen to demonstrate that the MIPs are able to selectively bind 4-NP from other interferences in complex matrices. Ebro river water is a complex sample due to the presence of high concentrations of humic acids and therefore represents an interesting test case. As expected, the clean-up step reduced the humic band considerably but the use of dichloromethane was insufficient to completely remove the humic acids and the analytes could not be quantified accurately. Hence it was decided to add Na_2SO_3 (10% w/v) to the sample (80 µl Na_2SO_3 per 20 ml of sample) since this gave cleaner chromatograms when Ebro river water was used in previous work [28]. Adding Na_2SO_3 did indeed decrease the humic band and enabled 4-NP to be quantified accu-rately. These results are shown in Figures 4 and 5 for P1 and P2, respectively. The recovery of 4-NP is similar to the recovery obtained under the same conditions with Milli-Q water. Therefore, Na_2SO_3 plays an important role when real water samples are analysed.

P1 and P2 were compared in the extraction of 4-NP from real water samples. As in the model study, when river water was analysed, P1 showed a slightly higher selectivity since the interaction with humic acids was higher with the P2 polymer.

Linearity with river water samples under the optimum conditions was tested using P2 as an example.

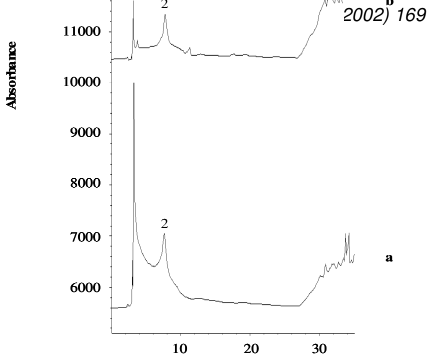

Figure 4. Chromatograms obtained by on-line MISPE with the non-covalent 4-NP imprinted polymer (P1) of 10 ml Ebro river water (pH 2.5) spiked at 10 µg/l with each phenolic compound. (a) With washing step using 0.2 ml of dichloromethane, and (b) With addition of Na_2SO_3 to the washing step. Peak designation, as per Figure 2.

Different samples of 20 ml volume spiked with 4-NP at concentrations between 100 and 1 µg l^{-1} and containing 80 µl of Na_2SO_3 per 20 ml sample, were pre-concentrated and a washing step with 0.5 ml of dichloro-methane applied. The response was checked in the range described earlier and good linearity was obtained with a determination coefficient (r^2) higher than 0.999. The repeatability for 20 ml of spiked (5µg l^{-1} of each component) river water, expressed as RSD (n=3), was 7%. The application of the imprinted polymers to on-line MISPE of real samples was demonstrated.

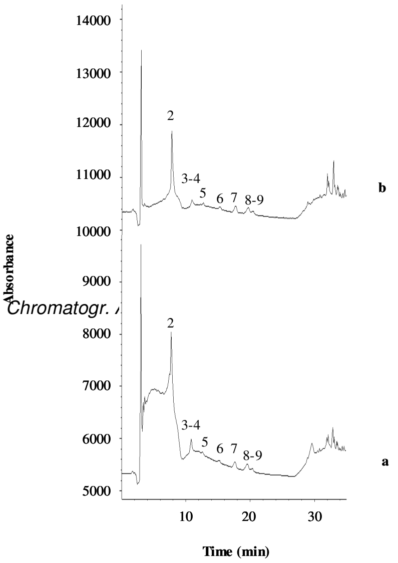

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Figure 5. Chromatograms obtained by on-line MISPE with the semi-covalent 4-NP imprinted polymer (P2) of 20 ml Ebro river water (pH 2.5) spiked at 5 µg/l with each phenolic compound. (a) With washing step using 0.5 ml of dichloromethane, and (b) With addition of Na_2SO_3 to the washing step. Peak designation, as per Figure 2.

CONCLUSIONS

The results demonstrated the practicality of the on-line coupling of MIPSE to liquid chromatography. Two approaches (non-covalent and semi-covalent) were tested for the MIPSE of 4-NP from water samples and differences in selectivity and recovery were observed. Whereas the non-covalent MIP was more selective, the semi-covalent one showed slightly higher recoveries of 4-NP. The application of the MIPSE procedure to determine 4-NP in the presence of other compounds in real water samples was demonstrated.

Acknowledgements

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2.1.2 *On-line solid-phase extraction with molecularly imprinted polymers to selectively extract substituted 4-chlorophenols and 4-nitrophenol from water*

ON-LINE SOLID-PHASE EXTRACTION WITH MOLECULARLY IMPRINTED POLYMERS TO SELECTIVELY EXTRACT SUBSTITUTED 4-CHLOROPHENOLS AND 4-NITROPHENOL FROM WATER

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Abstract

Three polymers have been synthesised using 4-chlorophenol (4-CP) as the template, following different protocols (non-covalent and semi-covalent) and using different functional co-monomers, 4-vinylpyridine (4-VP) and methacrylic acid (MAA). The polymers were evaluated to check their selectivity as molecularly imprinted polymers (MIPs) in solid phase extraction (SPE) coupled on-line to liquid chromatography. The solid phase extraction procedure using MIPs (MISPE), including the clean-up step to remove any interferences, was optimised. The 4-VP non-covalent polymer was the only one which showed a clear imprint effect. This MIP also showed cross-reactivity for the 4-chloro substituted phenols and for 4-nitrophenol (4-NP) from a mixture containing the eleven priority EPA (Environmental Protection Agency) phenolic compounds and 4-chlorophenol. The MIP was applied to selectively extract the 4-chloro substituted compounds and 4-NP from river water samples.

Keywords: 4-chlorophenols; 4-nitrophenol; Cross-reactivity; On-line solid-phase extraction; Molecularly imprinted polymer; Water sample

INTRODUCTION

Molecularly imprinted polymers (MIPs), which can be prepared by three different protocols [1,2], are highly crosslinked polymers synthesised in the presence of a template molecule. However in some cases, the MIP recognises not only the template in the rebinding step but also structurally related analytes [3-7]. This effect, known as cross-reactivity, is particularly interesting in environmental samples, since these may contain several structurally related compounds.

Nearly all the data published about MIPs as sorbents in solid-phase extraction (MISPE), has been generated in an off-

J. Chromatogr. A 995 (2003) 233

line mode [4,8-10]. Few applications have been developed using an on-line mode with the MISPE coupled to the liquid chromatography (LC) [2,3,11-14] and only some of them have used only one imprinted pre-column [2,12,13] which offers significant advantages in terms of the ease of instrumentation. Moreover, MISPE has been mainly applied to biosamples in which several drugs [15-18] have been determined and there are far fewer applications relating to the extraction of analytes in environmental samples [2,3,5,11,12,14].

The aim of this work was to synthesise and evaluate three different polymers potentially selective for 4-CP and to demonstrate how selective the MIP can be in the analysis of real water samples by on-line SPE-LC.

EXPERIMENTAL

Reagents and standards

The chemicals used for the polymer syntheses were 4-chlorophenol (4-CP), 4-vinylpyridine (4-VP), methacrylic acid (MAA) and ethylene glycol dimethacrylate (EGDMA) from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium) and HPLC grade acetonitrile (ACN) from Rathburn Chemicals (Walkerburn, U.K.). The monomers and the AIBN were purified prior to use via standard procedures in order to remove stabilisers. The monomer derivatised template, 4-chlorophenyl methacrylate, was synthesised according to a protocol described in the literature [19].

The HPLC-grade solvents were provided by either Rathburn Chemicals or SDS (Peypin, France) and the water collected from a Millipore water purification system (Milli-Q water). The acetic and hydrochloric acids were from Probus (Badalona, Spain) and the dichloromethane (DCM) from SDS. The structurally related phenolic pollutants used to investigate the selectivity of the polymers were the eleven priority EPA phenolic compounds and 4-chlorophenol itself. Phenol (Ph), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP), 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2-nitrophenol (2-NP) 2,4-dimethylphenol (2,4-DMP), 4-chloro-3-methylphenol (4-C-3-MP), 2-methyl-4,6-dinitrophenol (2-M-4,6-DNP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP), were all supplied by Aldrich, except for PCP which was from Jansen Chemie (Geel, Belgium).

Instrumentation

The polymers were firstly evaluated in analytical columns to check the imprinting effect. The 15 x 0,46 cm i.d. stainless steel HPLC columns were slurry packed with the ground polymer particles (25-38 μm) using an air-driven fluid pump (Haskel) with acetone as the solvent at 2500 p.s.i. An SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

The equipment used for the MISPE study, which has been described in some previous works [2,12], was on-line coupled to a LC system. This system has two pumps and enables the compounds retained on the pre-column to be eluted only by the organic solvent of the mobile phase [12]. The analytical column was a 25 x 0.4 cm i.d. Tracer Extrasil ODS2, 5 µm, supplied by Teknokroma (Barcelona, Spain).

Preparation of the Imprinted Polymers

Polymers P1 and P2 were prepared by the non-covalent approach. The pre-polymerisation mixture for P1 comprised 4-CP (2.02 mmol) as the template molecule, 4-VP (8.07 mmol) as the functional monomer, the cross-linking monomer EGDMA (40.36 mmol) and the initiator AIBN (1.13 mmol) dissolved in ACN, (11.25 ml) in a 25 ml thick-walled glass tube. The poly-merisation procedure followed and the subsequent treatment to obtain the small particles suitable for the SPE evaluation is described in a previous work [12].

The P2 pre-polymerisation mixture comprised 4-CP (2.14 mmol) and MAA (8.58 mmol) as the template molecule and the functional monomer respec-tively, the cross-linker EGDMA (42.88 mmol) and the initiator AIBN (0.94 mmol) dissolved in ACN (11.75 ml) in a 25 ml thick-walled glass tube. The synthetic procedure followed during the poly-merisation step was the same as for P1 polymer.

Two reference, non-imprinted polymers, B1 and B2, which did not contain any template, were prepared analogously to P1 and P2 using the same protocols respectively.

Polymer P3 was prepared by the semi-covalent approach using 4-chlorophenyl methacrylate (2 mmol) as a template, styrene (6 mmol) as an additional functional co-monomer [2], the cross-linker EGDMA (40 mmol) and the initiator AIBN (0.88 mmol) dissolved in ACN (7.08 ml) in a 25 ml thick-walled glass tube. P3 was synthesised in the same way as P1 but, on this occasion, when the monolith obtained was dried, crushed and ground, the polymer was subjected to a different protocol which is described in a previous work [2] for a 4-NP MIP. A final elemental micro-analysis demonstrated that the tem-plate had been successfully removed from the polymer.

Chromatographic Conditions

For the chromatographic evaluation of the polymers, ACN/acetic acid (99.7/0.3 *(v/v)*) was used as the mobile phase at 0.5 ml min^{-1}. The injection volume was 20 µl, the UV detector wavelength was 280 nm and the analyses were performed at room temperature.

For the MISPE experiments the mobile phase consisted of Milli-Q quality water, acidified to pH 2.5 with acetic acid, as solvent A and ACN (containing 1% (*v/v)* acetic acid) as solvent B. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was 20-30% B from 0-30 min, 100% B at 32 min and then isocratic elution for 2 min. The oven temperature was set at 65 ºC and all compounds were detected at 280 nm, except for PCP which was detected at 302 nm.

On-Line MISPE Procedure

The polymers were packed into stainless-steel pre-columns in order to be evaluated via on-line MISPE. Prior to any injections they were washed with solvent B to verify that there was no residual template (4-CP) present.

For on-line MISPE the polymers were conditioned with 5 ml ACN and 2 ml acidified Milli-Q water with HCl (pH 2.5) at 3 ml min^{-1}. The spiked water sample (adjusted to pH 2.5) was applied to the conditioned pre-column, and the polymer then washed with 0.1 ml (P1) of DCM and 4 ml Milli-Q water (pH 2.5). The retained analytes were desorbed using solvent B alone and in the back-flush mode [12].

Real samples were filtered through a 0.45 µm filter and adjusted to pH 2.5 before MISPE.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

The analytical columns packed with the polymers were first washed on-line with acetonitrile/acetic acid (99.7/0.3 *(v/v)*), to eliminate interfering compounds from the synthesis. For their evaluation, 10 mg l^{-1} solutions of Ph, 4-NP, and 4-CP were injected as test analytes onto the columns. The three compounds gave different retention times on the non-imprinted blank column, for this reason, the data obtained in these analyses was normalised by calculating the Normalised Retention Index (RI) [12]. Table 1 shows the K' values for the test compounds in the column and the corresponding RI values for P1. Table 1 shows the K' values for the test phenolic compounds in the column and the corresponding RI values for P1. 4-NP and Ph give rise to smaller RI values than 4-CP thus the imprinting effect seems to be verified.

The imprinting effect in P2 and P3 was evaluated by on-line MISPE since the RI values were not conclusive.

On-Line MISPE

To investigate the selectivity of the polymers for 4-CP when this phenol was present with the 11 priority EPA phenolic compounds in a water sample, an extraction step was developed.

Table 1. Capacity Factors (*K'*) and Normalised Retention Indices (RI) obtained from HPLC evaluation of polymer (P1)

Analyte	K’ (MIP)	K' (control)	RI
Ph	0.56	0.47	0.81
4-NP	1.57	1.17	0.93
4-CP	1.01	0.71	1

The P1 polymer was first evaluated and 10 ml of spiked (10 µg l^{-1} of each analyte) Milli-Q water, previously adjusted with HCl to pH 2.5, was passed through the sorbent.

All twelve compounds were retained on the MIP when a clean-up step was not performed. However, to favour the selectivity of the polymer, a clean-up step with an organic solvent was included. DCM was selected as the washing solvent and different volumes were tested (0.1, 0.2 and 0.3 ml). When 0.1 ml of DCM was used, not only did the template (4-CP) remain strongly bound to the polymer in the imprinted sites but also all the 4-chloro-substituted phenolic compounds (4-C-3MP, 2,4-DCP, TCP and PCP) as well as the 4-NP (Figure 1). This behaviour can be explained by the fact that this polymer shows cross-reactivity. The other compounds were washed straight off the MIP. These results, which are shown in Table 2, prompted further experiments and the applicability to real water samples of this polymer. In the case of B1, when 0.1 ml of DCM was used all the phenols were stripped off the polymer, including 4-CP, which indicated that there were no selective binding sites.

P2 and P3 polymers were evaluated in the same way as P1 but no clear imprinting effect was obvious, since even with 0.1 ml of DCM the 4-CP was almost completely eluted from P2 and P3. This behaviour was expected since in the chromatographic evaluation no clear imprinting effect was established. MIPs selective for nitrophenols or chlorophenols synthesised using 4-VP as functional monomer show higher recoveries than those obtained using MAA [2] since there is much better interaction between the phenolic aromatic ring of the analytes and the 4-VP. Moreover, the basic pyridine group in the 4-VP functional monomer is able to form stronger non-covalent bonds with acidic compounds (nitro and chlorophenols) than the carboxylic acid derived from MAA.

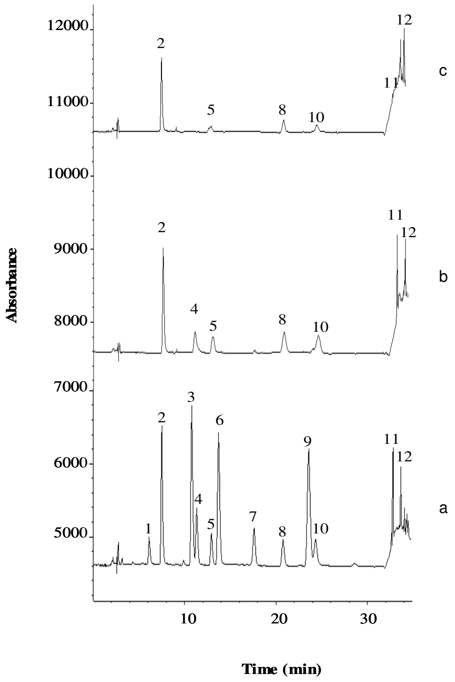

Figure 1. Chromatograms obtained by on-line MISPE with the 4-VP non-covalent 4-CP imprinted polymer (P1) of 10 ml standard solution (pH 2.5) spiked at 10 $\mu g\ l^{-1}$ with each phenolic compound. (a) Without washing step, and (b, c) with washing step using 0.1 and 0.3 of dichloro-methane, respectively: (1) Ph, (2) 4-NP, (3) 2,4-DNP, (4) 2-CP, (5) 4-CP, (6) 2-NP, (7) 2,4-DMP, (8) 4-C-3-MP, (9) 2-M-4,6-DNP, (10) 2,4-DCP, (11) 2,4,6-TCP, (12) PCP.

Table 2. Recoveries (%) obtained by washing the non-covalent 4-CP imprinted polymer P1 with different volumes of dichloromethane following the pre-concentration of 10 ml of a standard solution spiked at 10 µg l^{-1} for each analyte [a].

Analyte	Volume CH_2Cl_2 (ml)			
	0	0.1	0.2	0.3
Ph	59	-	-	-
4-NP	73	55	47	38
2,4-DNP	71	[b]	-	-
2-CP	82	[c]	-	-
4-CP	68	53	41	13
2-NP	74	-	-	-
2,4-DMP	71	-	-	-
4-C-3-MP	65	64	61	33
2-M-4,6-DNP	66	-	-	-
2,4-DCP	67	58	50	17
2,4,6-TCP	50	44	25	-
PCP	23	24	13	11

[a] RSDs were lower than 9% in all instances (n= 3)

[b,c] Co-eluted compounds

The effect of the sample volume on the recovery was tested and 20 ml of sample was percolated. When the clean-up step was carried out with 0.1 ml of DCM, the recovery decreased considerably and larger sample volumes were therefore not tested. From these results, a volume of 10 ml was selected as the optimum value for further experiments.

MISPE of Real Water Samples

An application of the imprinted polymer (P1) in the MISPE was developed with Ebro river water sample to show how selective the MIP can be with real water. Polar phenols can not be accurately quantified at low levels when they are in Ebro water because the complex matrix usually contains humic acids which appear as a broad band at the beginning of the chromatogram interfering in the quantification of the most polar compounds. However, when this MIP was used as selective sorbent in SPE the humic band was completely removed with a clean-up step with 0.1 ml of DCM (Figure 2) and without adding Na_2SO_3 as required with other sorbents [2,20]. The analytes selectively retained on the MIP were then accurately quantified. The recovery values for the retained compounds were nearly the same as in Milli-Q water when the clean-up step was not performed and when it was carried out under the optimum conditions (0.1 ml of DCM).

To check the linear range, 10 ml of river water, which did not contain any phenolic compounds, were spiked with the eleven priority EPA phenolic compounds and 4-CP at concentrations between 100 and 1 µg l^{-1}, were then pre-concentrated and a washing step with 0.1 ml of DCM was applied. Since the polymer showed cross-reactivity, linearity was checked for all the retained compounds in the MIP.

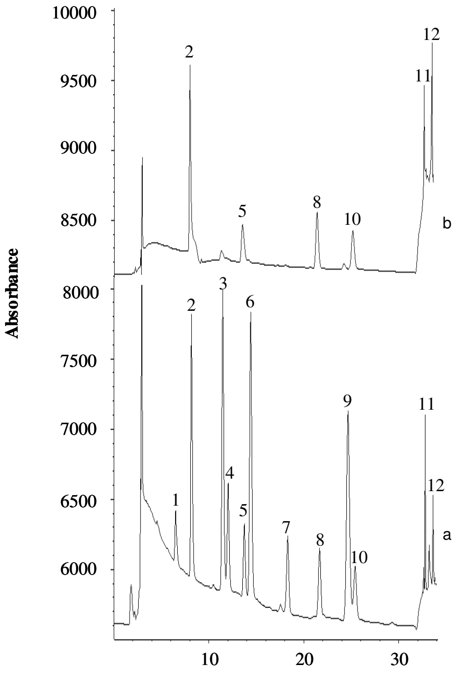

Figure 2. Chromatogram obtained by on-line MISPE with the 4-VP non-covalent 4-CP imprinted polymer (P1) of 10 ml Ebro river water (pH 2.5) spiked at 10 µg l^{-1} with each phenolic compound. (a) Without washing step and (b) with washing step using 0.1 ml of dichloromethane. Peak designa-tion, as per Figure 1.

Good linearity was obtained for all six phenols, with a determination coefficient (r^2) higher than 0.999. The repeatability for 10 ml of spiked (10 µg l^{-1} of each component) river water, expressed as RSD (n=3), was lower than 11%. The application of the imprinted polymers to on-line MISPE of real samples has therefore been demonstrated.

CONCLUSIONS

A polymer prepared using 4-VP as functional monomer and following non-covalent imprinting protocol (P1) showed an imprinting effect for all 4-chloro-substituted phenolic compounds and 4-NP since this MIP showed cross-reactivity. The selectivity of the MIP, which was evaluated in SPE coupled on-line to liquid chromatography, and the cross-reactivity were evident when a clean-up step with DCM as washing solvent was performed. An application of the MIPSE procedure in real water samples was developed to demonstrate its applicability. All the retained compounds gave a linear response.

Acknowledgements

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2.2 EXTRACCIÓ EN FASE SÒLIDA DE NAFTALENS SULFONATS EN MOSTRES D'AIGUA DE RIU MITJANÇANT UN POLÍMER AMB EMPREMTA MOLECULAR

Arran dels resultats obtinguts en els estudis anteriors, on s'ha comprovat que és possible sintetitzar MIPs selectius per extreure compostos de característiques polars d'una mostra aquosa, es va proposar la síntesi d'un nou MIP que fos capaç d'extreure compostos iònics i de mida més gran com són els naftalens sulfonats.

En aquest apartat es presenta la síntesi i l'aplicació d'un MIP empremtat amb l'àcid 1-naftalensulfònic (1-NS) i amb la 4-VP com a monòmer funcional, ja que com es va comprovar en els dos treballs anteriors, aquest monòmer dóna lloc a interaccions més fortes quan el *template* presenta aromaticitat. No obstant, trobar el porogen apropiat per a dur a terme aquesta síntesi va ser força complicat, ja que en ser un compost tan polar era gairebé insoluble en la majoria de solvents orgànics emprats normalment per a la síntesi de MIPs (apolars i apròtics). Es va observar però que en acetonitril, aquest *template* sí que era soluble i per tant inicialment es va escollir aquest solvent com a porogen. No obstant, quan s'afegia el monòmer funcional a aquesta barreja, es formava immediatament un precipitat blanc, fet que indicava que hi havia una forta interacció entre l'àcid 1-NS i la 4-VP. La formació d'aquest precipitat no era adient per a poder continuar afegint els altres reactius necessaris per a la polimerització i per aquest motiu l'ACN va quedar descartat com a porogen. Un altre solvent de constant dielèctrica elevada i apròtic que es va provar va ser la dimetilformamida (DMF), però l'1-NS era poc soluble en aquest solvent.

Basant-nos en els resultats obtinguts en uns estudis previs desenvolupats per Haupt *et al.*[1] i Baggiani *et al.* [2], els quals havien preparat els respectius MIPs emprant una barreja de metanol/aigua (MeOH/H_2O) com a porogen, es va intentar sintetitzar el MIP en aquest medi. La quantitat d'aigua a emprar en el nostre cas venia determinada per la miscibilitat amb l'agent entrecreuant (EGDMA). Aquesta proporció va ser finalment de 4 parts de MeOH per cada part d'H_2O (4:1) i es va observar que l'ordre d'addició dels reactius era important per tal d'aconseguir una total solubilització de la molècula *template*. L'ordre òptim a seguir en aquest cas va ser afegir primer el template, després l'aigua, a continuació la 4-VP i per últim el MeOH.

El protocol d’empremta molecular va ser el no covalent. La polimerització en solució es va iniciar amb radiació UV (50 Hz) durant 24 hores a 5ºC. Després d’aquest període, el polímer obtingut es va introduir en un bany d’aigua a 60ºC durant 24 hores més per tal d’assegurar que s’obtenia un monòlit rígid i estable. El polímer de control es va sintetitzar simultàniament però en absència de la molècula *template*.

Per tal d’avaluar l’efecte d’empremta molecular del MIP abans de ser emprat com a sorbent en SPE, es va intentar realitzar una avaluació cromatogràfica, però no es va obtenir cap resultat ja que, degut a la forta interacció que presentava l’analit amb el MIP, resultava impossible eluir-lo.

Així doncs, per tal de poder optimitzar el procés de MISPE sense cap tipus de restricció pel que fa al solvent, es va optar per començar utilitzant el procés de MISPE fora de línia. En aquest cas es va empaquetar una xeringa d’extracció amb 200 mg del sorbent que es trobava contingut entre dos fritats. Després d’optimitzar l’etapa d’acondicionament i pas de la mostra, es va veure que per dur a terme l’elució era necessari emprar una barreja de MeOH/H_2O (4:1) amb un 10% d’hidròxid sòdic (NaOH) 1M. Per tal d’evitar problemes de degradació amb la columna analítica i degut a que aquesta composició de fase mòbil no era l’adient per dur a terme la separació cromatogràfica dels compostos, es va continuar treballant en aquest sistema fora de línia.

Tot i que el MIP havia estat sintetitzat en presència d’aigua, i que això podia suposar un inconvenient per la formació de les interaccions específiques durant la polimerització, es va comprovar com el MIP presentava un bon efecte d’empremta molecular i que a més a més també presentava una forta reactivitat creuada de manera que permetia extreure selectivament una barreja de vuit naftalens sulfonats i disulfonats (NSs) amb diversos grups funcionals (amino- i nitro-) d’una mostra de 1000 ml d’aigua Milli-Q amb bones recuperacions per a tots els compostos després d’una etapa de neteja amb un solvent orgànic

(MeOH/piridina (99:1)). Aquesta barreja de compostos es perdia gairebé per complet del polímer de control després de l'etapa de neteja.

Per tal de demostrar que aquest grup de NSs quedaven retinguts al MIP mitjançant interaccions selectives, es va preparar una mostra d'aigua Milli-Q on a més dels 8 NSs també es van afegir altres compostos polars com l'oxamil, metomil, el 4-NP, el 2,4-DNP, la bentazona i l'àcid 4-cloro-2-metil-fenoxi acètic i es va comprovar com després d'una etapa de neteja, en aquest cas amb MeOH, els NSs quedaven retinguts al 100% mentre que la resta d'analits s'eluien totalment. Aquest fet justifica que el grup sulfònic (SO_3^-) juga un paper molt important en el reconeixement molecular de l'analit a extreure i que per tant aquestes interaccions selectives estan basades en interaccions iòniques entre la 4-VP i el SO_3^-.

Finalment, aquest MIP es va utilitzar en l'extracció de vuit NSs en aigua de riu. En aquest cas, el volum de mostra analitzat van ser 500 ml.

Amb aquest MIP s'aconsegueix extreure una família de compostos d'elevada polaritat, els quals són difícils d'extreure amb sorbents convencionals. Aquests compostos normalment s'extreuen mitjançant SPE amb parell iònic amb l'inconvenient que sovint altres substàncies iòniques presents a la mostra interfereixen en l'extracció dels compostos d'interès [3]. Per tant, les recuperacions i el volum de ruptura disminueixen considerablement [4]. En aquest estudi queda demostrat, que també és possible extreure aquests compostos de grans volums de mostra amb bones recuperacions sense necessitat d'utilitzar un parell iònic quan es treballa amb aquest MIP com a sorbent.

Els resultats obtinguts en aquest estudi s'inclouen en el treball que s'adjunta a continuació i que ha estat publicat a la revista *Journal of Chromatography A 1047 (2004) 175.*

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2.2.1 *Molecularly imprinted solid-phase extraction of naphthalene sulfonates from water*

MOLECULARLY IMPRINTED SOLID-PHASE EXTRACTION OF NAPHTHALENE SULFONATES FROM WATER

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Abstract

A new polymeric sorbent synthesised by exploiting molecular imprinting technology has been used to selectively extract naphthalene sulfonates (NSs) directly from aqueous samples. In the non-covalent molecular imprinting approach used to prepare this polymer, 1-naphthalene sulfonic acid (1-NS) and 4-vinylpyridine (4-VP) were used as a template molecule and functional monomer, respectively, and both dissolved in a mixture of methanol/water (4:1) as porogen together with the cross-linker ethylene glycol dimethacrylate.

The new non-covalent molecularly imprinted polymer (MIP) prepared in aqueous environment was used as a sorbent in solid-phase extraction (SPE) to selectively extract a group of naphthalene mono- and disulfonates. When one litre of a standard aqueous solution, which contained a mixture of eight NSs, was percolated through the SPE cartridge, all the NSs were retained on the MIP because of the cross-reactivity of the polymer. Recoveries were higher than 80% for all the compounds even after a clean-up step with methanol (MeOH). The MIP was also used to analyse water from the Ebro river.

Keywords: Water analysis; Cross-reactivity; Molecularly imprinted polymers; 1-Naphthalene sulfonic acid

INTRODUCTION

J. Chromatogr. A 1047 (2004) 175

Naphthalene sulfonates (NSs) are aromatic compounds that show low biodegradability, high polarity and high toxicity. All these properties make them potentially hazardous for the environment in particular surface and ground waters are of concern with regard to drinking water quality [1]. The presence of these pollutants in the environment can be attributed mainly to their use in the chemical industry [2]. For these reasons, NSs have been investigated for some time, and several analytical methods have been developed to determine them in environmental water [3-7]. Reversed-phase ion-pair liquid chromatography has been the most used method [1,2,6-10]. The characteristics of NSs make the interactions of these analytes with reversed-phase columns too weak to separate them effectively; consequent-ly, other retention mechanisms using an ion-pairing agent have to be exploited [1].

In environmental samples naphthalene sulfonates are normally present at low concentrations and an enrichment step is required for their quantification. Ion-pair solid-phase extraction (ion-pair SPE) is the most commonly used pre-concentration procedure for these analytes. However, ion-pair SPE suffers from some drawbacks in respect of aqueous samples since other ionic species can compete in the formation of the ion-pair, thus interfering with the SPE process [2]. Consequently the recoveries and therefore the break-through volumes decrease considerably [11]. Thus it would be very advantageous to work without using an ion-pairing reagent in the SPE procedure and this would be possible using selective SPE materials such as molecularly imprinted polymers (MIPs) [12-15].

The main advantage of this class of sorbents, compared to conventional sorbents, is the inherent molecular selectivity of the sorbent [16], which enables MIPs to extract one specified compound from a complex mixture of compounds, although in some cases the MIP shows cross-reactivity and is able to recognise also structurally related analytes [17-22].

In this study, a new non-covalently MIP has been prepared and evaluated for the extraction of a group of NSs from water samples. Non-covalently MIPs are usually synthesised in apolar organic solvents because polar solvents can disrupt hydrogen bonding interaction between the template and the functional monomer. Moreover, if ionic interactions are also established polar-protic solvents can also disrupt them. However, it has been previously reported [23-26] that some times is also possible to use a mixture of polar solvents as porogen (such as MeOH/water) when a polar compound is used as template in which a combination of the hydrophobic effect and ionic interactions will be the responsible in the molecular recognition step. To our knowledge this is the first time that an imprinted polymer has been synthesised using 1-naphthalene sulfonic acid as a template molecule using a mixture of methanol/water (4:1) as porogen. Thus, in the present paper a water compatible MIP (aqua-MIP), which is quite helpful when using aqueous samples, has been synthe-sised.

EXPERIMENTAL

Reagents and standards

The chemicals used for the polymer syntheses were 1-naphthalenesulfonic acid dihydrate (1-NS) from Avocado Research Chemicals (Lancarshire, England), 4-vinylpyridine (4-VP) and ethylene glycol dimethacrylate (EGDMA) from Aldrich (Steinheim, Germany) and 2,2'-azobisisobu-tyronitrile (AIBN) from Acros Organics (Geel, Belgium). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers. The AIBN was recrystallised from acetone and the methanol dried over molecular sieves.

The HPLC-grade methanol was provided by SDS (Peypin, France) and the water collected from a Millipore water purification system (Milli-Q water). The phosphoric acid was from Probus (Badalona, Spain) and the disodium hydrogen phosphate, the sodium dihydrogen phosphate and the tetra-butylammonium bromide (TBA) were from Panreac (Barcelona, Spain), Probus and Fluka (Buchs, Switerland), respectively. The latter were to prepare the mobile phase.

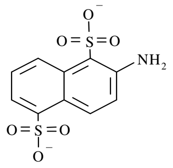
Other reagents used to modify the pH of the sample were hydrochloric acid (37%) from Probus, sodium hydroxide (NaOH) from Prolabo (Fontenay, France), triethylamine (TEA) from Aldrich and pyridine, which was used in the washing step, from Fluka.

The structurally related naphthalene sulfonate pollutants (Figure 1) used to investigate the selectivity of the polymers were 2-naphthylamine-1-sulfonic acid (1-NS-2-NH_2), 1-naphthol-4-sulfonic acid sodium salt (1-NS-4-OH), naphthalene-2-sulfonic acid sodium salt (2-NS), naphthalene-1,5-disulfonic acid disodium salt (1,5-NDS), 2-naphthylamine-1,5-disulfonic acid disodium salt (1,5-NDS-2-NH_2), naphthalene-2,7-disulfonic acid diso-dium salt (2,7-NDS), 1-naphthol-3,6-disulfonic acid disodium salt (3,6-NDS-1-OH); all sourced from Fluka. Standard solutions of each compound at a concentration of 1000 mg l^{-1} were prepared in Milli-Q quality water. The 2-NS, was solubilised in Milli-Q water/methanol 70:30 (*v/v*).

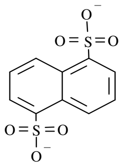
Other compounds such as naphthalene, phenol (Ph), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP), 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2-nitrophenol (2-NP), 2,4-dimethylphenol (2,4-DMP), oxamyl, methomyl, bentazone and 4-chloro-2-methyl-phenoxy acetic acid (MCPA), supplied by Aldrich and Fluka, were used to check the selectivity of the MIP for other polar compounds.

Instrumentation

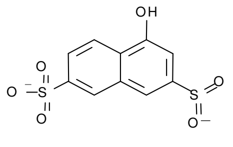
The liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and a SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan)



1,5-NDS-2-NH_2



1,5-NDS



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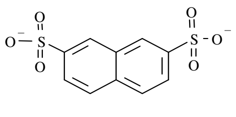
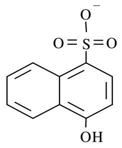
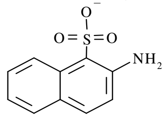


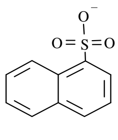
2,7-NDS



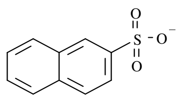
1-NS-4-OH



1-NS-2-NH_2



1-NS



2-NS

Figure 1. Chemical structures of the naphthalene sulfonates (NSs) used to probe the selectivity of the MIP.

The injection loop volume was 20 µl and the analytical column was a 25 x 0.4 cm i.d. Tracer Extrasil ODS2, 5 µm, supplied by Teknokroma (Barcelona, Spain).

Preparation of the Imprinted Polymer

The imprinted polymer was prepared by the non-covalent approach. The molar ratio template: functional monomer: cross-linker was (1:4:20) in which 1-NS (2.28 mmol) was the template molecule, 4-VP (9.12 mmol) was selected as the functional co-monomer, EGDMA (45.6 mmol) was the cross-linking monomer and AIBN (1.00 mmol) was the initiator. Due to the high polarity of the template, a mixture of methanol/water (4:1 *v/v*) (13.33 ml) was used as porogen. Other solvents were also evaluated as possible porogens to avoid the presence of water during the polymerisation (note: water can disrupt hydrogen bonding or ionic interactions between templates and the functional monomers), but the template was not completely soluble in any of them. All the components were mixed in a 25 ml thick-walled glass tube fitted with a screw cap and added in the following order: template, water, 4-VP, methanol, EGDMA and AIBN. This solution was cooled in an ice bath, sparged with oxygen-free nitrogen for five minutes, sealed under nitrogen and then left in a cooled bath at 5 ºC for 24 hours while irradiated with a 50 Hz Black-Ray Non-UV Semi-conductor Inspection Lamp, Model B 100 AP. The polymer obtained was then left in a water bath at 60ºC for 24 hours to maximise the cure and to ensure formation of a rigid, stable monolith. The MIP was crushed mechanically, ground and wet-sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 µm suitable for the MISPE evaluations.

A reference, non-imprinted polymer (NIP), which did not contain the template, was prepared in parallel with the imprinted polymer using the same synthetic protocol.

Chromatographic Conditions

The mobile phase used in the MISPE experiments was a mixture of two solvents [2]. Solvent A, the aqueous component, contained 8 mM of disodium hydrogen phosphate, 8 mM of sodium dihydrogen phosphate and 7 mM of TBA in Milli-Q water and the pH was adjusted to 6.5 with phosphoric acid. Solvent B was methanol (MeOH). The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was 20-50% B from 0-25 min, and then isocratic elution for 5 min. The oven temperature was set at 40 ºC and all compounds were detected at 230 nm.

MISPE Procedure

The MISPE study was developed in an off-line mode using a solid-phase extraction manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump. 200 mg of polymer suspended in Milli-Q water was packed into a 6 ml SPE cartridge. Prior to any extraction the polymer was washed with an eluting mixture of MeOH/water (4:1) containing 10% NaOH (1M) until no more residual template (1-NS) was eluted from the polymer. For the MISPE experiments the polymer was conditioned with 15 ml of MeOH and 15 ml of acidified Milli-Q water (pH 2.3). The required sample volume (adjusted to pH 2.3 with HCl) was applied to the conditioned cartridge and the polymer then washed with 20 ml of MeOH. The retained analytes were desorbed using 5 ml of a mixture of MeOH/water (4:1) containing 10% NaOH (1 M) and 20 µl samples injected onto the analytical column.

River samples were filtered through a 0.45 µm filter prior to any experiment.

RESULTS AND DISCUSSION

To evaluate the imprinting effect of a MIP, a chromatographic evaluation is often performed. For this purpose, a chromatographic column (15 x 0.46 cm i.d.) was packed with the MIP by following a standard in-house procedure [12].

Several different mobile phases were investigated including MeOH, MeOH/water (95:5), MeOH/water (80:20), MeOH/water/pyridine (92.5:2.5:5), MeOH/pyridine (95:5), MeOH/acetic acid (80:20) and MeOH/TBA (80:20). However, no chromatographic peaks were obtained when a sample containing 10 mM of 1-NS was injected onto the column, which suggested that the analyte was still retained on the MIP and that the affinity was strong under these particular chromatographic conditions. Therefore, the imprinting effect was evaluated by SPE instead.

MISPE

Two hundred milligrams of the polymer was packed into a polyethylene cartridge. Off-line SPE was chosen as the preferred method of analysis because in this way the most effective solvents could be used to elute the analyte from the MIP. Thus, a mixture of naphthalene mono- and disulfonic acid compounds, some of which are hydroxyl and/or amino substituted was percolated through the cartridge.

Several parameters were optimised in the SPE procedure. Firstly, the sample pH was investigated to optimise the interactions between the analytes and the MIP in the loading step. Accordingly, the samples were prepared in Milli-Q water and acidified Milli-Q water (pH 2.3). 25ml of these solutions spiked with 5 mg l^{-1} of the NSs mixture were percolated through the MIP. The recovery of each analyte in this loading step was determined by measuring the concentration of the analyte in the aqueous solution eluted from the cartridge. The compounds were strongly retained on the MIP when the sample was prepared in acidified Milli-Q water; however, when the sample was in pure Milli-Q water, very little of each analyte was retained (30%). This observation is presumably a reflection of the fact that electrostatic interactions between the analytes and the pyridyl group residues in the polymer would be expected to have higher affinity at the lower pH value.

The elution step was therefore optimised for samples delivered in acidic water. 5 ml volumes of different elution solvents such as MeOH/water (4:1), MeOH/water (4:1) containing 10% of TEA, and MeOH/water (4:1) containing 10% of NaOH (1M) were tested. The analytes were not eluted when MeOH/water was used as the eluting solvent. However, when 10% of TEA or 10% of NaOH (1M) were added to MeOH/water (4:1), the compounds were eluted with high recoveries although TEA gave rise to slightly distorted peaks for all analytes. To avoid this distortion MeOH/water (4:1) containing 10% of NaOH (1M) was chosen as the elution solvent. Thus, not only did the distortion disappear with this elution solvent but the recoveries of the analytes were also slightly higher than those obtained using TEA.

To evaluate the imprinting effect of the MIP a clean-up step was included. 5 ml volumes of dichloromethane (DCM), acetonitrile (ACN), methanol (MeOH), and a mixture of MeOH/water (4:1) were tested as possible washing solvents, but no clean-up effect was observed because the recoveries of 1-NS and indeed all the NSs present in the sample were the same as SPE without a clean-up. This suggests that the MIP shows cross-reactivity effect for the naphthalene sulfonates. When 1% and 5% of NaOH (1M) were each added to ACN, MeOH and Milli-Q water, the recovery values decreased slightly for all compounds.

Identical experiments were performed using the NIP, and it was found that the analytes were not washed straight off the polymer during the clean-up step with any of the organic wash solvents mentioned previously. Therefore, to demonstrate that the MIP was imprinted, a wash with the most effective solvent was performed. For this purpose, pyridine was added to the most polar solvent (MeOH) since pyridine will compete with the functional monomer (4-VP) for the analyte. Different amounts of pyridine were used (1%, 5% and 10%) and the best results obtained when the cartridge was washed with 2 ml of MeOH/pyridine (99:1) (Table 1).

Table 1. Recoveries (%) of naphthalene sulfonic acid compounds using the 1-NS imprinted polymer and the non-imprinted polymer, either without a clean-up step or with a clean-up step involving 2 ml of MeOH/pyridine (99:1) at different sample volumes of a standard solution.

Analyte	Sample Volume (ml)											
	1-NS						NIP					
	No clean-up			Clean-up			No Clean-up			Clean-up		
	5	500	1000	5	500	1000	5	500	1000	5	500	1000
1,5-NDS-2-NH_2	107	106	104	100	80	82	88	52	14	17	14	10
1,5-NDS	107	101	103	98	84	84	93	61	25	16	14	11
3,6-NDS-1-OH	85	86	87	77	73	72	88	52	25	8	10	7
2,7-NDS	119	112	110	98	87	86	83	58	27	14	16	10
1-NS-4-OH	104	98	105	55	50	50	83	37	16	17	3	16
1-NS-2-NH_2	105	100	82	70	59	60	84	29	9	13	6	5
1-NS	117	90	85	51	50	54	80	44	14	14	16	6
2-NS	115	110	102	51	62	65	88	54	17	18	15	8

^{a}RSDs were lower than 13% in all instances (n= 3)

The addition of pyridine to the MeOH disrupted the non-specific interactions established between the analytes and the NIP so that the NSs were almost completely eluted upon the introduction of the washing step. When the same washing step was applied to the MIP, the recoveries decreased slightly for the retained NSs because the non-specific interactions were also disrupted; however, all the NSs were still retained on the polymer because of the specific interactions between the analytes and the polymer. This also confirms that the MIP shows cross-reactivity effect.

The selectivity of the MIP was also evaluated in other ways. For this purpose, a second group of polar pollutants was also added to the sample. Thus 25 ml of acidified Milli-Q water (pH 2.3) containing 2 mg l^{-1} of the mixture of NSs and 2 mg l^{-1} of a mixture of several polar compounds (oxamyl, methomyl, Ph, 4-NP, 2,4-DNP, bentazone and MCPA) was percolated through the cartridge. All the com-pounds, except for oxamyl and methomyl, were retained on the MIP in the loading step.

The MIP was washed with an organic solvent to remove the non-selectively bound compounds. MeOH was chosen as the washing solvent because, as explained earlier, the NSs were retained on the MIP when this solvent was applied. However, it was expected that if the mixture of polar compounds were not selectively retained on the MIP, then MeOH would be able to elute them. A 10 ml volume of MeOH was sufficient to strip off the phenols from the polymer while the

NSs still remained strongly bound to the MIP (Figure 2).

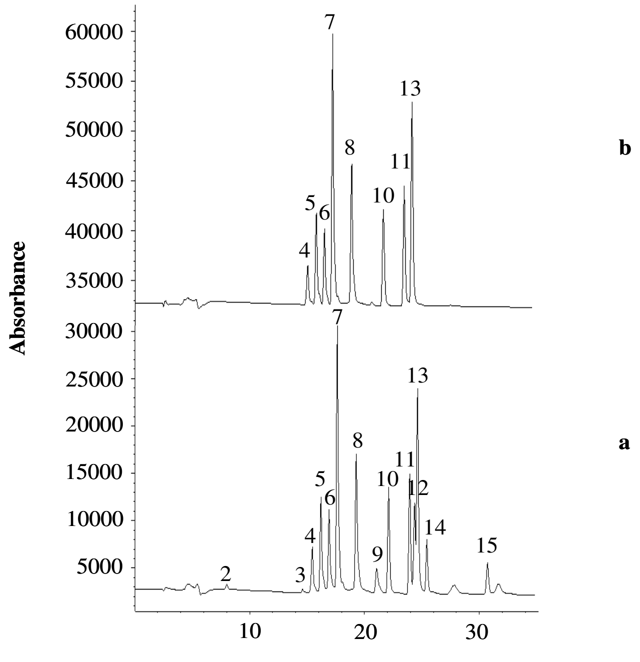


Figure 2. Chromatogram obtained by MISPE of 25 ml of a standard solution (pH 2.3) spiked at 2 mg l^{-1} with each polar and naphthalene sulfonic acid compound: (a) without washing step, and (b) with washing step using 10 ml of methanol: (1) oxamyl, (2) methomyl, (3) Ph, (4) 1,5-NDS-2-NH_2, (5) 1,5-NDS, (6) 3,6-NDS-1-OH, (7) 2,7-NDS, (8) 1-NS-4-OH, (9) 4-NP, (10) 1-NS-2-NH_2, (11) 1-NS, (12) 2,4-DNP, (13) 2-NS, (14) bentazone, (15) MCPA.

Table 2. Recoveries (%) of naphthalene sulfonic acid compounds using the 1-NS MIP when different sample volumes of a standard solution were extracted and a clean-up step with 20 ml of MeOH was applied.

Analyte	Sample volume (ml)		
	100	500	1000
1,5-NDS-2-NH_2	107	106	104
1,5-NDS	107	105	103
3,6-NDS-1-OH	85	86	87
2,7-NDS	109	102	100
1-NS-4-OH	104	98	105
1-NS-2-NH_2	105	100	82
1-NS	107	90	85
2-NS	105	100	102

J. Chromatogr. A 1047 (2004) 175

[a]RSDs were lower than 5% in all instances (n= 3)

However, 20 ml of MeOH was chosen finally as the optimum volume because the recoveries for the NSs were the same as when 10 ml MeOH was used (Table 2) and a higher volume of MeOH would be expected to be more effective in removing a matrix of polar compounds when real river water is analysed.

Other polar compounds such as 2-CP, 4-CP, 2-NP and 2,4-DMP and an apolar aromatic compound such as naphthalene were also tested. As expected, when a 25 ml sample (spiked with 2 mg $^{l-}$1 of polar compounds and naphthalene) was percolated, all the compounds were removed during the clean-up step with 10 ml of MeOH because they could not establish the required specific interactions with the MIP because they each lack the necessary SO_3^- group.

Thus it is clear that the SO_3^- group in the NSs plays an important role in the molecular recognition step by establishing ionic interactions with the 4-VP residues in the MIP. This also accounts for the cross-reactivity effects and this probably explains why after the washing step all the NSs are also retained on the MIP.

The recovery of the compounds from different sample volumes was studied also for both polymers (Table 1). The recoveries for the MIP and the NIP were nearly the same when a 5 ml sample as pre-concentrated and no clean-up performed. However, these values were completely different when a clean-up step was included and the polymers washed with 2 ml MeOH/pyridine (99:1). When 25 ml of sample was passed through the MIP, (data not shown) the recoveries for the NSs in the MIP were still the same, while recoveries decreased in the NIP (the recovery of 1-NS was only 56%). At higher sample volumes (500 ml and 1000 ml) the differences between both polymers were yet higher, even in the absence of a clean-up step.

MISPE of Real Water Samples

The performance of the MIP in the extraction of NSs from real river water samples was investigated. For this purpose, Ebro river water was chosen. From this kind of water sample, a broad band due to the presence of humic acids appears in the middle of the chromatogram. However, the selectivity of the MIP for the NSs allows us to selectively extract and quantify them after a suitable clean-up procedure.

One thousand milliliters of an Ebro river water sample was percolated through the MIP. However, the recoveries decreased significantly for all the retained analytes even without the clean-up step due to the humic acids and other substances present in the sample matrix affect the retention of the compounds. Therefore 500 ml was chosen as the sample volume. With this volume the recoveries were nearly the same as in Milli-Q water for all the compounds except for 1-NS-2-NH_2 where the recovery was 50%.

When the MIP was washed with 20 ml of MeOH (Figure 3) the band due to the humic acids was reduced and the recoveries of the NSs were nearly the same as those obtained without the washing step, except for the 1-NS-2-NH_2 (16%) (Table 3). This step was also performed using 2 ml of MeOH/pyridine (99:1) as the washing solvent. However, the recoveries were lower than those obtained using MeOH and the humic band did not decrease significantly with respect to the blank. For this reason, the experiments to test the useful linear range of the application in river water analysis were carried out using MeOH as the washing solvent.

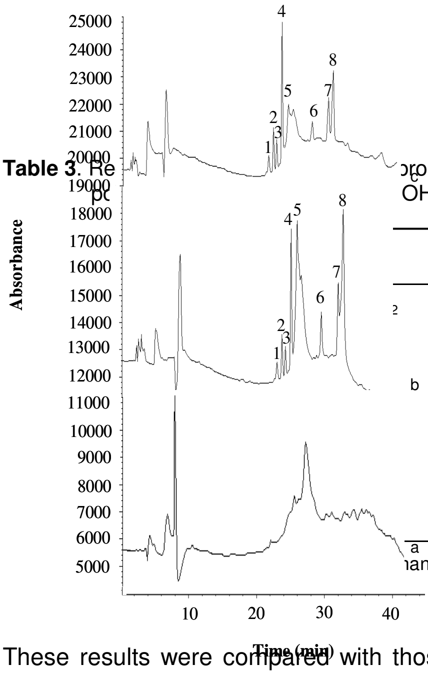


Figure 3. Chromatogram obtained by MISPE of 500 ml Ebro river water (pH 2.3). (a) Blank of Ebro river water, (b) Ebro river water spiked at 10 µg l^{-1} with each NS compound, and (c) Ebro river water spiked at 10 µg l^{-1} with each NS compound and a washing step using 20 ml of MeOH. Peak designation: (1) 1,5-NDS-2-NH_2, (2) 1,5-NDS, (3) 3,6-NDS-1-OH, (4) 2,7-NDS, (5) 1-NS-4-OH, (6) 1-NS-2-NH_2, (7) 1-NS, (8) 2-NS.

Table 3. Re ro river water spiked at 50 µg l^{-1} using the 1-NS imprinted p OH.

500 ml (50 µg l^{-1})
72
94
78
106
81
16
72
93

an 11% in all instances (n= 3)

These results were compared with those obtained by Alonso et al. [27] using a highly cross-linked sorbent (Isolute ENV+), in which an ion-pair reagent was neither added to the sample in the loading step. The recoveries for the NSs with more than one SO_3^- with the Isolute ENV+ are better than those using other sorbents such as Lichrolut EN or graphitized carbon black, but are lower than those using the 1-NS MIP. Only 150 ml of groundwater sample was percolated and the recoveries for some of the NSs were still low (1,5-NDS-2-NH_2 in 27%, 1,5-NDS in 42%, and 3,6-NDS-1-OH in 71%).

To test the performance of the method in river water samples, the linear range under the optimum conditions was determined. 500 ml a sample, which did not contain any NSs, was spiked with the eight NSs at concentrations between 100 and 5 µg l^{-1} and their recovery examined. A washing step with 20 ml of MeOH was applied. Good linearity was obtained, with a

determination coefficient (r^2) higher than 0.9998 for all compounds. The repeatability for 500 ml of spiked (10 µg l^{-1} of each component) river water, expressed as RSD (n=3), was lower than 11%. Although the lowest level tested was 5 µg l^{-1}, this level could be easily decreased by evaporating the 5 ml of the elution solvent to 500 µl with a stream of nitrogen coupled with the use of a fluorescence detector.

The application of the MIP synthesised using 1-NS as template to selectively extract a group of NSs from river water samples has therefore been demonstrated.

CONCLUSIONS

A non-covalently molecularly imprinted polymer using 1-NS as a template has been synthesised in a mixture of methanol/water (4:1) and applied for the first time to the MISPE of eight NSs. This Aqua-MIP, which shows cross-reactivity, was able to extract selectively the NSs from a mixture of polar compounds, such as phenols, pesticides and naphthalene (apolar compound with a close structure to NSs), when a clean-up step with MeOH was used. 200 mg of the MIP used as SPE material was sufficient to extract 1000 ml of standard solution and 500 ml of river water with high recoveries for all the NS studied even after a clean-up step. This sorbent prepared in aqueous environment has better retention for NSs than most commercially available sorbents. Finally, the method was validated with river water and good linearity and repeatability demonstrated.

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2.3 EXTRACCIÓ D'ANTIINFLAMATORIS DE MOSTRES D'AIGUA DE RIU I D'ORINA MITJANÇANT POLÍMERS AMB EMPREMTA MOLECULAR

Com a conseqüència dels bons resultats obtinguts en l'anterior estudi, on una molècula d'elevada polaritat i mida superior a la dels compostos fenòlics havia estat emprada com a molècula *template*, en aquest apartat s'inclouen dos estudis en els quals es presenta la síntesi de dos nous MIPs empremtats amb ibuprofen i naproxen, dos antiinflamatoris que presenten una estructura molecular una mica més gran que la dels compostos fenòlics. Per una altra banda també cal destacar que l'interès en la determinació d'aquests compostos ha augmentat molt darrerament ja que són compostos molt emprats i estan inclosos en el que es coneix com a contaminants orgànics emergents.

Tant l'ibuprofen, el naproxen, així com el també antiinflamatori ketoprofen, han estat utilitzats en diversos estudis com a *template* [1-5]. No obstant, en la majoria d'aquests treballs el MIP obtingut és emprat com a sorbent en columnes cromatogràfiques per tal d'assolir la separació enantiomèrica dels compostos esmentats. De tots aquests estudis, només el dut a terme per Haginaka *et al.* [5], va ser aplicat a l'SPE. En aquest treball [5], cal assenyalar que el sorbent utilitzat és un RAM empremtat molecularment. No obstant, l'ibuprofen no pot ser extret selectivament degut al sagnat que aquest MIP presenta. Com a alternativa, utilitzen un RAM empremtat amb naproxen per a l'extracció de l'ibuprofen aprofitant la reactivitat creuada que el polímer presenta.

Els MIPs empremtats amb l'ibuprofen i amb el naproxen que es presenten en aquest apartat, es van preparar via no covalent mitjançant una polimerització convencional en solució. Un cop obtinguts els MIPs amb els respectius polímers de control, es van avaluar cromatogràficament per tal d'obtenir una idea sobre la selectivitat que presentava cada MIP pel respectiu *template* i també per altres compostos d'estructura similar

Degut als valors de reactivitat creuada que presenta el MIP empremtat amb l'ibuprofen, aquest MIP es va aplicar com a sorbent per a l'extracció selectiva d'un grup de quatre antiinflamatoris (naproxen, fenoprofen, diclofenac sòdic i ibuprofen) en mostres d'aigua de riu i de depuradora, ja que en no ser completament eliminats aquests compostos en les plantes de tractament d'aigües poden ser transferits al medi.

Després del procés d'optimització de la MISPE, el qual es va realitzar fora de línia amb la posterior tècnica cromatogràfica (HPLC), el MIP es va aplicar a l'extracció d'una mostra que contenia la mescla de quatre antiinflamatoris descrita prèviament i es va comprovar com aquest MIP permetia extreure'ls amb bones recuperacions fins i tot després d'haver preconcentrat un gran volum de mostra (1000 ml) i d'haver realitzat una etapa de neteja amb el corresponent solvent orgànic. La reactivitat creuada que presenta el MIP va afavorir que els quatre compostos quedessin retinguts després de l'etapa de neteja.

En aquest estudi cal destacar la gran capacitat que presenta aquest MIP ja que fins al moment, són molt pocs els MIPs sintetitzats que permeten extreure directament a través del propi MIP grans volums de mostra. A banda del polímer preparat prèviament en el nostre grup de recerca emprant l'1-NS com a *template* i el present MIP empremtat amb l'ibuprofen, es coneix també un altre estudi desenvolupat per Zhu *et al* [6] en el qual es va preparar un MIP selectiu per a un grup de sulfonilurees, les quals s'extreien de 1000 ml de mostres d'aigua de diversos tipus.

El MIP preparat amb l'ibuprofen com a *template* es va aplicar també a l'extracció dels quatre antiinflamatoris en aigües de depuradora, però en aquest cas el volum de mostra es va disminuir a 250 ml degut a la càrrega orgànica present en aquest tipus de mostra. Les recuperacions i la selectivitat obtingudes per aquest volum eren comparables a les de l'aigua del riu Ebre.

Degut als bons resultats obtinguts aplicant els MIPs a l'extracció de compostos en mostres ambientals, es va decidir ampliar el camp d'aplicació. Amb aquest objectiu, es va aplicar el MIP empremtat amb naproxen a l'extracció d'aquest compost en mostres d'orina humana. Els NSAIDs són els analgèsics més utilitzats arreu del món i s'ha comprovat com el consum continuat d'aquestes substàncies pot produir efectes adversos.

El procés de MISPE en aquest cas també va ser dut a terme fora de línia. El procés d'optimització es va dur a terme en aigua Milli-Q i degut a que el MIP mostrava una gran afinitat pel naproxen, tot i la polaritat d'aquest medi, l'aplicació de la mostra d'orina es va passar directament a través del cartutx del MIP. Després d'una etapa de neteja, només el naproxen quedava enllaçat en les cavitats del MIP mentre que les interferències de la matriu de la mostra, així com altres antiinflamatoris també presents (entre ells l'ibuprofen), eren eliminats. Fins al moment, els estudis de MISPE aplicats a l'extracció de compostos en fluids biològics on aquests siguin aplicats directament al MIP són minoritaris, ja que en la majoria de casos la mostra es dilueix amb un tampó o amb un solvent orgànic per tal de disminuir el contingut en aigua i així afavorir les interaccions entre l'analit i els grups funcionals del MIP.

Així doncs, en extreure selectivament només al naproxen es va optimitzar de nou el gradient de separació per tal de reduir el temps d'anàlisi. En aquestes noves condicions es van determinar la linealitat, la repetibilitat i el límit de detecció del mètode desenvolupat.

Els dos treballs que s'adjunten a continuació inclouen els resultats obtinguts en aquests estudis. En el primer treball (apartat 2.3.1) s'inclou la síntesi i aplicació del MIP empremtat amb l'ibuprofen, que ha estat acceptat per a la seva publicació a la revista *Journal of Science Separation*. La síntesi i aplicació corresponent al MIP empremtat amb el naproxen s'inclou en un segon treball (apartat 2.3.2) i ha estat publicat a la revista *Journal of Chromatography B 813 (2004) 137.*

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2.3.1 *Selective enrichment of anti-inflammatory drugs from river water samples by solid-phase extraction with a molecularly imprinted polymer*

SELECTIVE ENRICHMENT OF ANTI-INFLAMMATORY DRUGS FROM RIVER WATER SAMPLES BY SOLID-PHASE EXTRACTION WITH A MOLECULARLY IMPRINTED POLYMER.

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Abstract

This article describes the synthesis of a molecularly imprinted polymer by a non-covalent protocol in which ibuprofen was used as a template molecule. The polymer was evaluated chromatographically and it was seen that the MIP showed cross-reactivity. Subsequently, when this polymer was used as sorbent in solid-phase extraction it was possible to selectively extract a mixture of non-steroidal anti-inflammatory drugs from aqueous samples when a clean-up step with dichloromethane was performed. The performance of the MIP was evaluated with river water and water from a waste water treatment plant and compared with the performance of a commercial Isolute ENV+ sorbent.

Keywords: molecularly imprinted polymer; solid-phase extraction; ibuprofen; river water; cross-reactivity;

Abbreviations used:

WWTP: waste water treatment plant
4-VP: 4-vinylpyridine
SPE: solid-phase extraction
ACN: acetonitrile
MIPs: molecularly imprinted polymers
NIP: non-imprinted polymer
NSAID: non-steroidal anti-inflammatory drug
Ph: phenol
RAM: restricted access material
4-NP: 4-nitrophenol
EGDMA: ethylene glycol dimethacrylate
2,4-DNP: 2,4-dinitrophenol
AIBN: 2,2'-azobisisobutyronitrile
DCM: dichloromethane
MCPA: 4-chloro-2-methyl-phenoxy acetic acid

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INTRODUCTION

In the last few years, the use of pharmaceutical compounds for human and veterinary applications has grown significantly. Pharmaceutical products reach waste water treatment plants (WWTP) mainly *via* urinary and fecal excretion and from pharmaceutical manufacturing discharges. These compounds have been recently classified as emerging organic pollutants and they are not totally eliminated in the WWTP, consequently they can reach surface and ground water samples. Due to the biological activity of these compounds, they are of great environmental concern and their concentrations in natural waters must therefore be quantified [1].

When pollutants in water are to be determined, a pre-concentration step before the chromatographic separation is needed because the pollutants are normally present at low concentrations. Solid-phase extraction (SPE) has become a very important technique for sample preparation in the environmental field, however with conventional SPE materials this technique shows some limitations, such as low recoveries (observed mainly when polar com-pounds are extracted from pre-dominantly aqueous solutions) and lack of selectivity (which is mainly a problem when a specific compound must be isolated from a real sample) [2]. In the last few years, several sorbents have been synthesised to overcome these drawbacks. Some of these sorbents have been used successfully for extracting polar compounds with high recoveries [3], however they are not selective for a specific analyte. For this reason, selective sorbents such as immuno-sorbents and molecularly imprinted polymers (MIPs) have also been developed and recently applied to the selective extraction of target analytes from water samples.

MIPs have been applied to extract several pharmaceutical actives in different sample matrices [4]. Some of these pharmaceutical compounds are the non-steroidal anti-inflammatory drugs (NSADIs), which are widely used to treat pain, inflammations or fever in human and veterinary medicines. Most of the MIPs synthesised using a NSAID as template molecule have been applied as stationary phases in liquid chromatography for enantiomeric sepa-rations [5-9] and there are only two papers published where a MIP, imprinted with a NSAID has been used as selective sorbents in SPE procedures (MISPE) [10,11]. One of these studies [10] was previously performed in our research group and the naproxen MIP prepared was applied to the selective extraction of this analyte in urine samples. Nevertheless, in the study developed by Haginaka *et al.* [11] a MIP was prepared following a tedious multi-step swelling and polymerisation procedure using naproxen and ibuprofen as templates to prepare two restricted access-molecularly imprinted materials (RAM-MIP). In this case, the ibuprofen RAM-MIP showed bleeding of the template in use, thus it was not possible to use this polymer for the extraction of ibuprofen from serum samples. Therefore, the naproxen RAM-MIP was used to determine ibuprofen.

The aim of the present study was to synthesise a non-covalently imprinted polymer, using ibuprofen as template molecule, and to apply this in SPE procedures. To our knowledge, this is the first time in which a MIP prepared following a standard protocol [12] has been imprinted using ibuprofen, and the polymer then used for the selective extraction of a mixture of NSAIDs from river water samples.

EXPERIMENTAL

Reagents and standards

The MIP synthesis was performed using (S)-ibuprofen from Fluka (Buchs, Switerland), 4-vinylpyridine (4-VP), and ethylene glycol dimethacrylate (EGDMA) from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers. The AIBN was recrystallised from acetone and the toluene dried over molecular sieves.

The HPLC-grade acetonitrile (ACN) was provided by SDS (Peypin, France), the water collected from a Millipore water purification system (Milli-Q water) and the phosphoric acid was from Probus (Badalona, Spain); all were used to prepare the HPLC mobile phase. Other reagents used to modify the pH of the sample were hydrochloric acid and acetic acid, both supplied by Probus.

The structurally related compounds (Figure 1) used to investigate the selectivity of the polymer were benzoic acid and some NSAIDs, such as naproxen and fenoprofen (both from Aldrich) and diclofenac sodium (from Sigma). Standard solutions of each compound at concentration of 1000 mg l^{-1} were prepared in ACN.

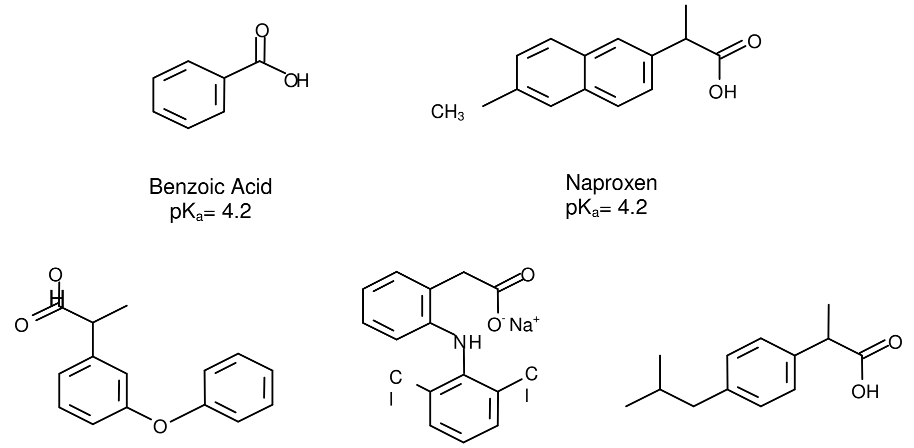
Other compounds, such as naph-thalene, phenol (Ph), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP), oxa-myl, methomyl, bentazone and 4-chloro-2-methyl-phenoxy acetic acid (MCPA), provided by Aldrich and Fluka, were used to check the selectivity of the MIP for other polar compounds.

Preparation of the Imprinted Polymer

The polymer, which was prepared by the non-covalent approach, was synthesised using 1.14 mmol (0.23g) of S-ibuprofen, 4.5 mmol (0.48g) of 4-VP, 22.80 mmol (4.52g) of EGDMA and 0.50 mmol (0.08g) of AIBN. All components were dissolved in 5.80 g (6.67 ml) of toluene (porogen) in a 25 ml thick-walled glass tube. This solution was cooled on an ice bath, sparged with oxygen-free nitrogen for five minutes, sealed under nitrogen and then left in a cool bath at –5 ºC for 24 hours for a UV polymerisation (50 Hz Black-Ray Non UV Semi-conductor Inspection Lamp, Model B 100 AP). The polymer obtained was then left in a water bath at 60ºC for 24 hours for a thermal cure to obtain a monolith.

The MIP was crushed, ground and wet-sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 µm suitable for the MISPE evaluations.

A reference, non-imprinted control polymer (NIP), which did not contain the template, was prepared simultaneously to the imprinted polymer using the same protocol.



Benzoic Acid
pK_a= 4.2

Naproxen
pK_a= 4.2

Fenoprofen
pK_a= 7.3

Diclofenac Sodium
pK_a= 3.9

Ibuprofen
pK_a=4.6

Figure 1. Chemical structures of the benzoic acid and the non-steroidal anti-inflammatory drugs used to probe the selectivity of the MIP.

Instrumentation

The MIP and NIP polymers were evaluated initially in HPLC analytical columns to check the imprinting effect. 15 x 0.46 cm i.d. stainless steel HPLC columns were slurry packed with the ground polymer particles (25-38 μm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an auto sampler and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

The MISPE study was developed in an off-line mode using a solid-phase extraction manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump. 200 mg of each polymer (MIP and NIP) suspended in MeOH was packed into an empty 6 ml polyethylene cartridge. The chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The loop for direct injection was 20 μl and the analytical column was a 25 x 0.4 cm i.d. Kromasil 100 C_{18}, 5 μm, supplied by Teknokroma.

Chromatographic Conditions

For the chromatographic evaluation, the MIP and the NIP were first washed with a mixture of acetonitrile/water/acetic acid (92.5:2.5:5 (*v/v/v*)) until no interfering compounds arising from the syntheses (template and un-reacted monomers) were detected. Then, 20 µl of the ibuprofen solution and 2 µl of the void marker (acetone) were injected onto the MIP and NIP. Acetonitrile was the mobile phase and the flow rate was set at 1 ml min^{-1} in isocratic mode. The UV detector wavelength was set at 224 nm.

A binary mobile phase with a gradient elution was used in the HPLC analysis for the MISPE experiments. Solvent A was Milli-Q water adjusted to pH 3 with phosphoric acid and solvent B was ACN. The gradient profile was 40-66.5% B from 0-21 min, 100% B at 27 min and then isocratic elution for 2 min. The oven temperature was set at 40 ºC and the flow-rate was 1 ml min^{-1}. Benzoic acid and naproxen were detected at 232 nm, whereas fenoprofen, diclofenac sodium and ibuprofen were detected at 224 nm.

MISPE Procedure

Prior to any extraction, the polymer was washed with a mixture of ACN/H_2O/acetic acid (60:30:10) until no residual template was present in the polymer. The cartridge was conditioned sequentially with 6 ml of ACN and 6 ml of acidified Milli-Q water (pH 3). The required sample volume (adjusted to pH 3 with HCl) was applied to the conditioned cartridge, and the polymer then washed with 3 ml of DCM. The analytes were eluted with 3 ml of ACN containing 1% of acetic acid. 20 µl of the eluent from the MISPE column was then injected onto the analytical column.

River samples were filtered through a 0.45 µm filter before any analysis.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

To check the imprinting effect in the MIP, 20 µl of 10 mM ibuprofen in ACN was injected onto the chromatographic columns containing the MIP and the NIP. From the retention times of the analyte and the void marker, the capacity factors for each column (k' ibuprofen$_{MIP}$= 2.35, k' ibuprofen$_{NIP}$= 1.59) and the imprinting factor (IF=1.48) were calculated. From these results, it was concluded that the MIP showed higher affinity for ibuprofen than the control polymer.

To investigate the selectivity of the MIP for other structurally related compounds, 20 µl of 10 mM naproxen in ACN was injected onto the MIP and NIP columns as a test analyte; the capacity factor of naproxen on each column was k' naproxen$_{MIP}$= 3.42 and k' naproxen$_{NIP}$= 2.58. The IF was 1.32 in this case, which is lower than that for the template (ibuprofen).

Ibuprofen and naproxen gave different retention times on the NIP, and for this reason the data were normalised by calculating the Normalised Retention Index (RI) [13]. The RI values were 1 and 0.90 for ibuprofen and naproxen, respectively. The imprinting effect is thus verified, but it should be noted that the MIP also shows some recognition for the naproxen molecule, which implies that the MIP shows cross-reactivity levels.

The chromatographic evaluation allo-wed us to confirm that the polymer was imprinted. For this reason, the MIP was then used in SPE procedures to selectively extract ibuprofen from water samples. The MISPE experiments also allowed us to demonstrate the cross-reactivity of the MIP to selectively extract a mixture of NSAIDs from river water samples.

MISPE

Several parameters must be optimised in MISPE. Thus, to favour the interactions between the analytes and the MIP, the loading step was first investigated. In this way, 10 ml of acidified Milli-Q water (pH 3) spiked with 15 µg l^{-1} of naproxen and 30 µg l^{-1} of the other structurally related compounds (benzoic acid, fenoprofen, diclofenac sodium and ibuprofen) was percolated through the MIP. The concentrations of analytes measured in the aqueous solution collected during this step, showed that all the compounds were strongly retained on the MIP (recoveries higher than 90%). When the pH of the sample was modified or the samples applied in organic solvents, the analytes were bound very weakly indeed to the polymer and were poorly retained [10].

For the elution step, ACN was used. With 5 ml of this solvent all the compounds were eluted completely from the MIP. However, when 1% of acetic acid was added to the ACN the volume of eluting solvent could then be reduced to 3 ml because acetic acid competes with the carboxylic acid groups of the analytes for binding to the 4-VP residues in the polymers. In these conditions, similar results were obtained; consequently ACN/acetic acid was used for further applications as eluting solvent.

It is well established that under aqueous loading conditions analytes are retained on a MIP mainly by non-specific, hydrophobic interactions [14,15]. It was suspected that the analytes were retained on the MIP by non-specific interactions because the same behaviour was observed when the sample was loaded through the NIP. Therefore, to remove the non-specifically bound analytes, a clean-up step with an organic solvent was included.

In the present work, and before each clean-up step, the cartridge was dried for 15 min by applying a vacuum [16-21]. DCM was used to perform the clean-up because if more polar solvents were used, the recovery for ibuprofen and the other NSAIDs decreased. It was found that 3 ml was sufficient volume to reveal a clear difference in the binding behaviour between the MIP and the NIP; all the analytes were strongly retained on the MIP with recoveries higher than 80%, while all them were nearly completely eluted from the NIP (except for benzoic acid and diclofenac sodium which were still retained). This behaviour of benzoic acid and diclofenac sodium was also observed in the previous study developed by our group for the naproxen MIP [10]. However, in this case to remove the retained diclofenac sodium the volume of DCM in the clean-up step should be increased to disrupt the non-specific interactions established with the 4-VP residues in the NIP, but in this situation the recovery for ibuprofen on the MIP was also decreased.

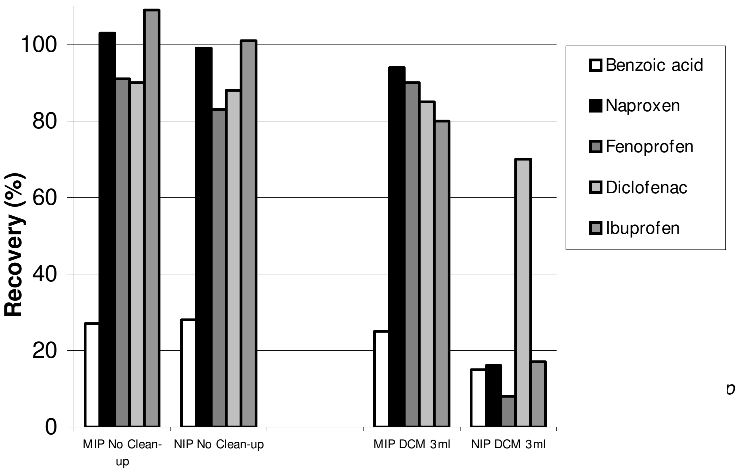
The selectivity of the MIP for other aromatic compounds was also evaluated. For this purpose, naphthalene and a group of polar pollutants including nitro- and chlorophenols, were added to the sample. Thus, when 10 ml of acidified Milli-Q water (pH 3) spiked with 15 µg l^{-1} of these compounds was percolated through the cartridge, all the compounds were retained on the MIP by non-specific, hydrophobic interactions. However, after the clean-up with 3 ml of DCM, these compounds were completely stripped off the MIP because they could not establish the specific interactions with the 4-VP residues in the polymer.

The recovery of the compounds at different sample volumes (100, 250, 500 and 1000 ml) was also studied. In this regard, the same SPE procedure described for a 10 ml of sample was applied. Thus, when 1000 ml of acidified Milli-Q water was pre-concentrated (Figure 2), the recoveries for the MIP and the NIP were nearly the same as when percolating 10 ml even after the clean-up with 3 ml of DCM, except for benzoic acid, which was almost lost (recovery about 25%) in both polymers.

From the MISPE study it could be concluded that not only was the MIP imprinted, as was implied by the chromatographic results, but also that the MIP showed cross-reactivity [14] for a group of NSAIDs structurally related to ibuprofen.

Analysis of River Water Samples

To demonstrate the feasibility of applying the MIP to the analysis of real samples, Ebro river water was analysed. For this purpose, 1000 ml of Ebro river water, acidified to pH 3 with HCl, was spiked with 0.5 µg l^{-1} of naproxen and 2.25 µg l^{-1} of benzoic acid and the other NSAIDs. When the sample was loaded on the MIP, all the compounds were completely retained. After drying the MIP, a clean-up step with 3 ml of DCM decreased slightly the broad band at the beginning of the chromatogram ascribed to the humic acids and disrupted the non-specific interactions established between compounds present in the river water and the MIP (Figure 3). As was expected, all the compounds were still retained on the MIP because of the selective interactions and the cross-reactivity of the polymer, although the recoveries (Table 1) were slightly lower than those obtained in Milli-Q water, which could be explained by the interferences present in the matrix sample.



ress)

Figure 2. Recovery of the molecularly imprinted polymer and the non-imprinted polymer after passing 1000 ml of acidified Milli-Q water spiked at 15 µg l^{-1} with naproxen and 30 µg l^{-1} of the other structurally related compounds (benzoic acid, fenoprofen, diclofenac sodium and ibuprofen) without and with a washing step with 3 ml of dichloromethane. (□) Benzoic acid, (■) naproxen, (■) fenoprofen, (■) diclofenac sodium, (■) ibuprofen.

Table 1. Recoveries (%) obtained by washing the non-covalently imprinted ibuprofen polymer (MIP) with 3 ml of dichloromethane following the pre-concentration of 1000 ml of Ebro river water spiked at 0.5 µg l^{-1} for naproxen and 2.25 µg l^{-1} for the other analytes (a).

Analyte	Volume of dichloromethane (ml)	
	0	3
Benzoic acid	21	17
Naproxen	65	60
Fenoprofen	64	43
Diclofenac sodium	90	86
Ibuprofen	103	80

[a] RSDs were lower than 5% in all instances (n= 5)

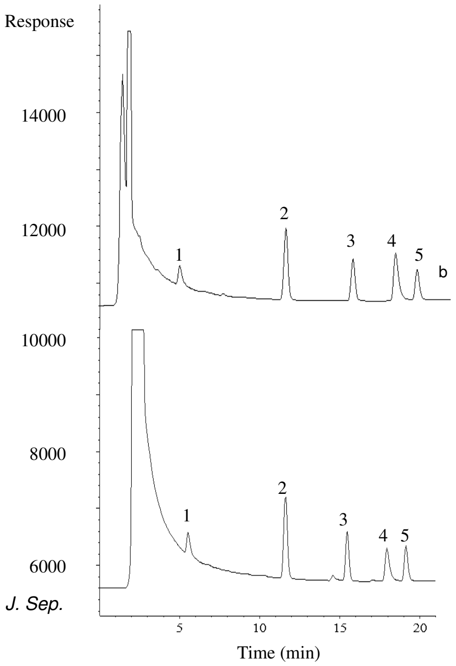


Figure 3. Chromatograms obtained by MISPE of 1000 ml of acidified Ebro river water (pH 3) spiked at 0.5 µg l^{-1} with naproxen and 2.25 µg l^{-1} with benzoic acid and the other NSAIDs. a) Without washing step; b) with a washing step using 3 ml of DCM. Peak assignation: (1) benzoic acid, (2) naproxen, (3) fenoprofen, (4) diclofenac sodium, (5) ibuprofen

Another source of water samples was also used to check the feasibility of applying the MIP widely to real water samples. WWTP effluent was then analysed. In this case, due to the high complexity of this kind of sample, 250 ml acidified to pH 3 and spiked with 4 µg l^{-1} of naproxen and 18 µg l^{-1} of the mixture of the other NSAIDs, was percolated through the MIP and the chromatogram is shown in Figure 4. The SPE protocol developed was applied and the recoveries were similar to those obtained for Ebro river water.

To compare the results from the MIP with those from a commercial cartridge, several experiments were performed with a cartridge containing 200 mg of Isolute ENV+ from Symta (Madrid, Spain) and treated under identical SPE conditions.

When 1000 ml of river water, acidified to pH 3 and spiked with 0.5 µg l^{-1} of naproxen and 2.25 µg l^{-1} of benzoic acid and the other NSAIDs, was percolated through the Isolute cartridge all the analytes were retained on it with recoveries greater than 70% (Figure 5). However, after a clean-up with 3 ml of DCM, fenoprofen and ibuprofen were retained less than 10%, diclofenac sodium could not be quantified due to the presence of a co-eluting impurity, and benzoic acid and naproxen were recovered in 90% and 50% respectively.

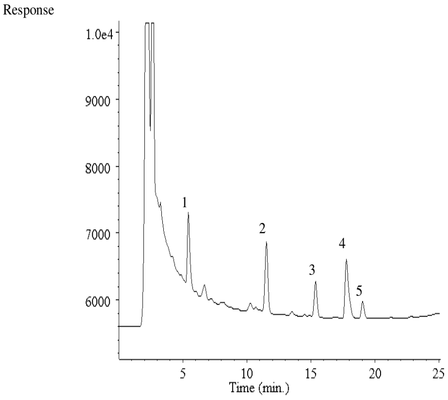


Figure 4. Chromatograms obtained by MISPE of 250 ml of acidified WWTP water (pH 3) spiked at 4 µg l^{-1} with naproxen and 18 µg l^{-1} with benzoic acid and the other NSAIDs when a clean-up step with 3 ml of DCM was performed. Peak assignation as per Figure 3.

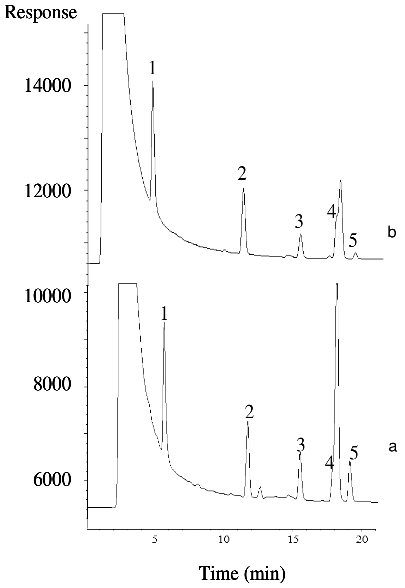


Figure 5. Chromatograms obtained after passing 1000 ml of acidified Ebro river water (pH 3) spiked at 0.5 µg l^{-1} with naproxen and 2.25 µg l^{-1} with benzoic acid and the other NSAIDs. a) through an Isolute ENV+ cartridge when a clean-up step was not performed. b) through an Isolute ENV+ cartridge with a clean-up using 3 ml of DCM. Peak assignation as per Figure 3.

It was also observed that the humic acid band at the beginning of the chromatogram was wider than that from the MIP, even after the clean-up.

Finally, the linearity of the method was evaluated. To check the linear range

1000 ml of Ebro river water, which did not contain any NSAIDs, was spiked with naproxen and the other NSAIDs at concentrations between 30 and 0.5 µg l^{-1} and pre-concentrated. A washing step with 3 ml of DCM was then applied. Good linearity was obtained with a determination coefficient (r^2) higher than 0.999. The repeatability for 1000 ml of spiked (0.5 µg l^{-1} of naproxen and 2 µg l^{-1} of the other NSAIDs) river water, expressed as RSD (n=5), was lower than 5%. The application of the imprinted polymer to the MISPE of river water samples has therefore been demonstrated.

CONCLUDING REMARKS

This study has shown that a polymer selective for ibuprofen can be successfully prepared *via* a non-covalent molecular imprinting approach. The MIP, which showed cross-reactivity levels, allowed us to selectively extract a mixture of NSAIDs from water samples. All analytes were strongly retained on the MIP with a linear response, even after a washing step with 3 ml of DCM. The performance of the MIP has been compared with a commercially available Isolute ENV+ sorbent. The results presented in the present paper demonstrate that not only are the recoveries between the MIP and the Isolute ENV+ comparable, but also that the MIP is more effective at removing the humic acid band at the beginning of the chromatogram when working with river water and water from a treatment plant and after a clean-up step with an organic solvent. Moreover, it has been demonstrated that it is possible to prepare an ibuprofen MIP following a non-covalent protocol to be used for the direct extraction from water of ibuprofen and a mixture of structurally related NSAIDs.

Acknowledgements

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2.3.2 A *new molecularly imprinted polymer for the selective extraction of naproxen from urine samples by solid-phase extraction*

A NEW MOLECULARLY IMPRINTED POLYMER FOR THE SELECTIVE EXTRACTION OF NAPROXEN FROM URINE SAMPLES BY SOLID-PHASE EXTRACTION

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Abstract

A non-covalent molecularly imprinted polymer (MIP) was synthesised using naproxen (a non-steroidal, anti-inflammatory drug (NSAID)) as a template molecule. The MIP was chromatographically evaluated to confirm the imprinting effect, and was then applied as a selective sorbent in solid-phase extraction (SPE) to selectively extract naproxen. After this study, the MIP was used to extract naproxen from urine samples; it was demonstrated that by applying a selective washing step with acetonitrile (ACN) the compounds in the sample that were structurally related to naproxen could be eliminated.

Keywords: Molecularly imprinted polymer; Solid-phase extraction; Naproxen; Human urine samples

INTRODUCTION

Non-steroidal, anti-inflammatory drugs (NSAIDs) are the analgesics used most commonly across the world today. They are used mainly to treat pain, inflammation and fever in animal and human species, although they can lead to severe toxic side-effects in cases of over dose or chronic abuse.

Several analytical techniques can be used to determine these analgesic compounds in biological samples [1]. In nearly all such techniques a suitable sample preparation step, such as liquid–liquid extraction (LLE) or solid-phase extraction (SPE), is an important pre-requisite to the analysis in order to clean and pre-concentrate the sample. In the last few years the clear advantages of SPE over the widely used LLE have made SPE the most important technique for sample preparation. However, typical SPE sorbents lack selectivity and this constitutes a problem when a selective extraction from a complex matrix has to be performed. To enhance the molecular selectivity in SPE, molecularly imprinted polymers (MIPs) [2] have been developed. MIPs allow the analyte of interest to be not only selectively extracted, but also to be pre-concentrated and interferents arising from the sample matrix to be removed simultaneously [3].

The majority of previous studies reported thus far in respect of naproxen imprints have focused on enantiomeric separations where the MIP was used as a chiral stationary phase in HPLC [4–7]. Moreover, in nearly all studies, the MIPs were prepared by following a long and tedious synthetic procedure called the multi-step swelling and polymerisation method [4,5,8]. Only in a study described by Haginaka and Sanbe [8], involving a restricted-access material in conjunction with a molecularly imprinted polymer (RAM-MIP), are the imprints used in an on-line SPE system coupled to an HPLC, for direct injection serum assay. In this particular study two MIPs were prepared, one using naproxen as the template and the other using ibuprofen. However, the naproxen RAM-MIP could not be used for assays of naproxen, and neither could the ibuprofen RAM-MIP be used for assays of ibuprofen, because in both cases leakage of the template from the imprinted polymers prevented accurate and precise assays of the drugs. The naproxen RAM-MIP was used finally to extract ibuprofen from rat plasma.

The aim of the present work was to demonstrate the feasibility of using MISPE for the selective clean-up and quantification of trace amounts of naproxen from human urine. To the best of our knowledge this is the first time that a MIP synthesised following a conventional non-covalent imprinting protocol using naproxen as the template molecule has been used as a sorbent in SPE of biological samples to extract naproxen selectively.

EXPERIMENTAL

Reagents and standards

For the polymer syntheses, the chemicals used were (*S*)-naproxen, 4-vinylpyridine (4-VP) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2’-azobisiso-butyronitrile (AIBN) from Acros Organics (Geel, Belgium), and HPLC-grade toluene from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use via standard procedures in order to remove stabilisers, and the solvent dried over 4 Å molecular sieves. The AIBN was recrystallised from acetone.

HPLC-grade acetonitrile (ACN) was provided either by Rathburn Chemicals or SDS (Peypin, France) and the water collected from a Milliporewater purification system (Milli-Q water). The acetic, hydrochloric and phosphoric acids were from Probus (Badalona, Spain) and dichloromethane (DCM) from SDS (Peypin, France).

The structurally related NSAIDs (Fig. 1) used to investigate the selectivity of the imprinted polymer were ibuprofen from

Fluka (Buchs, Switerland), diclofenac sodium from Sigma (Steinheim, Germany) and fenoprofen and benzoic acid from Aldrich. Standard solutions at 1000 mg l-1 for each compound were prepared in methanol.

Other compounds such as naphthalene, phenol (Ph), 4-nitrophenol (4-NP), 2,4-dinitrophenol (2,4-DNP), 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2-nitrophenol (2-NP), 2,4-dimethylphenol (2,4-DMP), 2-naphthylamine-1-sulfonic acid (1-NS-2-NH2), 1-naphthol-4-sulfonic acid sodium salt (1-NS-4-OH), naphthalene-2-sulfonic acid sodium salt (2-NS), naphthalene-1,5-disulfonic acid disodium salt (1,5-NDS), 2-naphthylamine-1,5-disulfonic acid diso- dium salt (1,5-NDS-2-NH2), naphthalene-2,7-disulfonic acid disodium salt (2,7-NDS), 1-naphthol-3,6-disulfonic acid disodium salt (3,6-NDS-1-OH), supplied by Aldrich and Fluka, were used to check the selectivity of the MIP for other aromatic compounds.

Preparation of the imprinted polymer

A non-covalent molecular imprinting approach was followed to prepare the MIP. The pre-polymerisation mixture comprised 0.26 g (1.14 mmol) of the template (S-naproxen), 0.48 g (4.56 mmol) of the functional monomer (4-VP), 4.52 g (22.8 mmol) of the cross-linking monomer (EGDMA) and 0.08 g (0.50 mmol) of the initiator (AIBN) dissolved in 6.66 ml of the porogen (toluene) in a 25ml thick-walled glass tube. This solution was cooled on an ice bath, sparged with oxygen-free nitrogen for 5 min, sealed under nitrogen and then left in a cool bath at -5 .C for 24 h for a UV polymerisation (50 Hz Black-Ray Non UV Semi-conductor Inspection Lamp, Model B 100 AP). The polymer obtained was then placed in a water bath at 60 ºC for 24 h for a thermal cure.

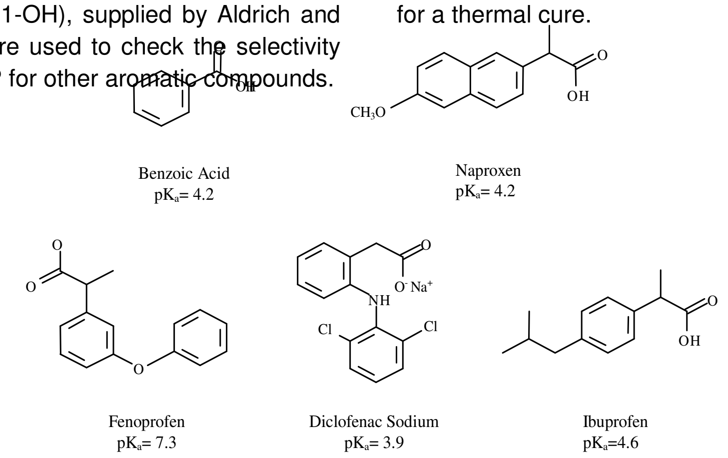


Figure 1. Chemical structures of the non-steroidal anti-inflammatory drugs used to probe the selectivity of the MIP.

The polymer monolith obtained was crushed, ground and wet-sieved using acetone to obtain polymer particles with diameters between 25 and 38 µm suitable for the chromatographic and MISPE evaluations.

A non-imprinted control polymer (NIP) was synthesised, in the absence of template, following the same procedure described above.

Instrumentation

The polymers were evaluated initially in analytical columns to confirm the imprinting effect. 15 cm × 0.46 cm i.d. stainless steel HPLC columns were slurry packed with the ground polymer particles (25–38 µm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an automatic injector and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

The MISPE study was developed in an off-line mode using a solid-phase extraction manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump. Two hundred milligrams of each polymer (MIP and NIP) suspended in MeOH was packed into a 6 ml polyethylene cartridge. The liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The injection volume was 20 µl and the analytical column was a 25 cm × 0.4 cm i.d. Tracer Extrasil ODS2, 5 µm, supplied by Teknokroma.

Chromatographic conditions

Before the chromatographic evaluation of the polymers, the chromatographic columns were washed with a mixture of acetonitrile/water/acetic acid (92.5:2.5:5 (v/v/v)) for about 20 h to eliminate interfering compounds arising from the synthesis (template and unreacted monomers).

For the chromatographic evaluation, 15 µl of 10 mM naproxen in ACN/acetic acid (99:1) and 2 µl of the void marker (acetone) were injected. The mobile phase was acetonitrile/acetic acid (99:1) and the flow rate was set at 1 ml min^{-1} in isocratic mode. The NIP was evaluated under identical chromatographic conditions. The UV detector wavelength was set at 232 nm and the analysis performed at room temperature.

The HPLC parameters for the MISPE experiments were as follows. The mobile phase was a mixture of two solvents: Milli-Q quality water adjusted to pH 3 with phosphoric acid (solvent A), and acetonitrile (solvent B). The flow rate of the mobile phase was 1 ml min-1 and the gradient profile was from 40 to 66.5% B in 21 min, to 100% B in 6 min and then isocratic elution for a further 2 min. The column temperature was 30 ºC.

MISPE conditions

Sample solutions (aqueous or urine adjusted to pH 3) were percolated through the cartridges which had been conditioned sequentially with 6 ml of ACN/H2O/acetic acid (60:30:10), 6ml of ACN and 6ml of Milli-Q water (pH 3). The polymers were then washed with an organic solvent (specified later) and the retained analytes desorbed with 3ml of ACN containing 1% of acetic acid. Twenty microliters of each sample was injected onto the analytical column.

Analysis of urine samples

Urine samples were kept in the freezer at -20ºC until their use. The urine was filtered through a 0.22 µm syringe filter before being applied to the MISPE cartridge.

RESULTS AND DISCUSSION

Chromatographic evaluation of the polymers

To confirm the imprinting effect the MIP was evaluated chromatogra-phically. ACN was chosen as the mobile phase, with 1% of acetic acid being added to this solvent to enable naproxen to be completely eluted from the MIP and to avoid long retention times and peaks with extensive tailing. From these results, the retention factors of naproxen in the MIP (k' $naproxen_{MIP}$= 2.29) and NIP (k' $naproxen_{NIP}$= 0.88) columns and the imprinting factor ($IF = k'_{MIP}/k'_{NIP}$) could be calculated (IF= 3). These values, taken together with the elution profiles, demonstrated that the MIP showed higher affinity for naproxen than the NIP and that the MIP was indeed imprinted.

The selectivity of the naproxen MIP for other structurally related analytes was also evaluated. For this purpose, ibuprofen, a NSAID with a structure similar to naproxen (Fig. 1), was injected onto the MIP and NIP columns as a test analyte. Thus, 15 µl of a 10mM solution of ibuprofen was injected onto each column and the retention factors calculated (k' $ibuprofen_{MIP}$= 1.00 and k' $ibuprofen_{NIP}$ = 0.51).

Naproxen and ibuprofen gave different retention times on the NIP, and for this reason the normalised retention index (RI) [9,10] was calculated to enable the *k* values of naproxen and ibuprofen to be compared. The RI value for the template (naproxen) is 1 by definition; for ibuprofen it was 0.75. From these results it can be concluded that the recognition of the template (naproxen) by the MIP is better than for ibuprofen, in spite of the similarity in their structures.

Exactly as one would expect for a naproxen imprint the chromatographic evaluation demonstrated clearly that the polymer was indeed imprinted. Thus, the MIP was taken forward and applied as an SPE sorbent to selectively extract naproxen from urine samples.

MISPE

The conditioning and the loading steps were first optimised, then 10 ml of a standard solution, spiked with 1.5 mg l^{-1} of naproxen and 3 mg l^{-1} of the other structurally related compounds (benzoic acid, fenoprofen, diclofenac sodium and ibuprofen), passed through the cartridge. The cartridge was condi-tioned with Milli-Q water at pH 3, and the sample was prepared in the same solvent. Under these conditions the compounds are in their non-dissociated (protonated) form (the p*K*a values are shown in Figure 1) and non-covalent interactions can be established between the hydroxyl groups of the NSAIDs and the 4-VP residues in the polymer. The analytes were strongly retained on the MIP with recoveries greater than 95% in all cases. When the sample was applied in organic solvents, or the pH of an aqueous sample was modified to neutral or basic, very little of each analyte was retained (R%< 30).

The next step was to optimise the elution solvent. Five aliquots of ACN, each of 1 ml in volume, were used initially to elute the compounds from the MIP. The concentration of naproxen and the other compounds were measured in each individual eluate fraction. Three milliliters of ACN was sufficient to elute all the compounds except for naproxen and diclofenac sodium which were still retained to some degree. For this reason, 1% of acetic acid was added to the ACN. As was expected given the chromatographic evaluation results, the addition of acetic acid as a polar modifier to ACN (total volume of ACN/acetic acid= 3 ml) enabled the efficient elution of naproxen and diclofenac sodium from the MIP (Table 1). Furthermore, no bleeding of the template from the MIP was observed.

It is well known that under aqueous loading conditions all the analytes present in the sample are retained on the MIP because non-specific hydrophobic interactions dominate. Thus, a washing (clean-up) step with an organic solvent was included to remove the non-specifically bound compounds from the MIP, such that only naproxen remained selectively bound through specific interactions with the imprinted binding sites. The behaviour of the NIP under these SPE conditions was also evaluated and compared with the MIP. Prior to the clean-up step, the cartridge was dried by applying a vacuum for 15 min [11–13]. The results arising from the optimisation of the elution solvent (Table 1) showed us that ACN was potentially a good washing solvent, thus 2 ml of ACN was used to reveal the imprinting effect (Figure 2). After this clean-up procedure was applied, while all the compounds were still retained on the MIP with recoveries close to 60% except for ibuprofen which was only 30% recovered, they were successfully stripped off the NIP (except for benzoic acid and diclofenac sodium). After the clean-up with ACN, benzoic acid and diclofenac sodium were still retained on the MIP and the NIP. As a result, it is necessary to use a greater volume of ACN to disrupt the non-specific interactions than was used for either ibuprofen or fenoprofen. DCM was also tested in the clean-up step. Thus, when 1 ml of DCM was applied, the recoveries measured for all compounds were nearly the same as those obtained in the absence of a washing step.

Table 1. Recoveries (%) obtained with the molecularly imprinted polymer (MIP) and the non-imprinted polymer (NIP) using acetonitrile or acetonitrile containing 1% of acetic acid as the elution solvent when 25 ml of a standard solution spiked at 55 μg l^{-1} for naproxen and 110 μg l^{-1} for the other analytes was pre-concentrated[a].

Analyte	Volume ACN (ml)						Volume ACN/AcOH (99:1) (ml)[b]		
	MIP	NIP	MIP	NIP	MIP	NIP	MIP	MIP	MIP
	1		2		3		1	2	3
Benzoic acid	-	13	39	50	95	90	30	80	104
Naproxen	-	14	36	82	60	105	50	70	99
Fenoprofen	20	22	39	90	98	99	60	90	101

Diclofenac	20	17	19	40	49	53	70	80	99
Ibuprofen	30	20	73	90	103	97	100	102	105

[a] RSDs were lower than 8% in all instances (n= 3)

[b] NIP values are not included because the recoveries were 100% in all instances

The volume of DCM was therefore increased to 3 ml and the imprinting effect was then revealed. Figure 2 shows the difference in behaviour between the MIP and the NIP. As can be clearly seen, the effect of using DCM was similar to that of ACN.

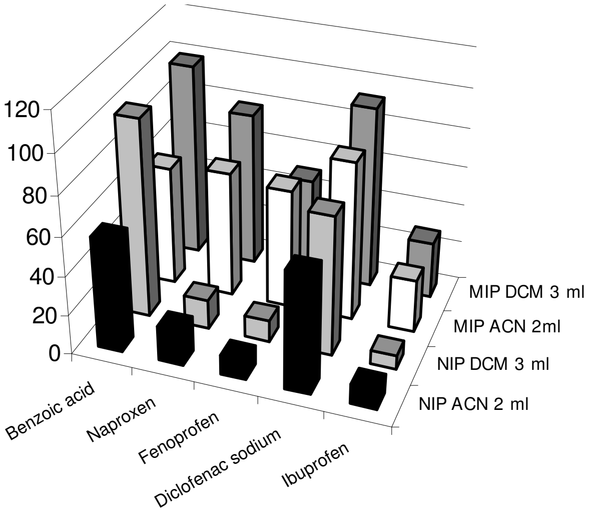


Figure 2. Selectivity of the molecularly imprinted polymer (MIP) and the non-imprinted polymer (NIP) after a washing step with 2 ml of ACN or, alternatively, with 3 ml of DCM.

To probe the selectivity of the MIP in the solvent used originally as the porogen (solvent memory effect) [11–14], a number of experiments were performed where several milliliters of toluene were applied. However, no imprinting effect was observed even after a clean-up step involving 6 ml of toluene, because toluene is insufficiently polar to elute analytes from such polymers.

It has been shown that ACN and DCM have similar effects as washing solvents on the MIP and the NIP. Both solvents allow us to demonstrate that the polymer was imprinted as was expected given the earlier chromatographic evaluation results. Moreover, when 3 ml of DCM or 2 ml of ACN was applied, other aromatic compounds (3 mg l^{-1}) such as phenols and naphthalene sulfonates (previously described in Section 2) were also completely removed from the MIP.

The performance of the MIP in selectively extracting naproxen from real samples was also evaluated with the polymer being applied to the extraction of naproxen from human urine.

Analysis of urine samples

Drug free urine samples, obtained from healthy volunteers, were used in this study. To determine NSAIDs at the levels normally found in humans, a urine volume of 25 ml was chosen because there were no differences, in terms of recovery, between percolating 10 ml samples and 25 ml samples. Matrix interferences affecting molecular recognition and the use of ACN or DCM in the clean-up step are discussed below.

The urine samples could be loaded directly onto the MIP cartridge because binding of naproxen to the MIP was complete in water. For this reason the urine was not diluted using organic solvents or buffers [15,16], but was acidified to pH 3 using HCl.

Twenty-five milliliters of urine spiked with 55 µg l^{-1} of naproxen and 110 µg l^{-1} of the other NSAIDs and benzoic acid was percolated through the MIP after conditioning of the polymer.

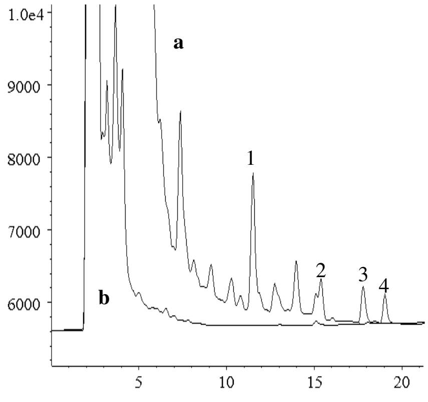
After drying the cartridge for 15 min, the effect of using DCM as washing solvent was first checked. When 3 ml of this solvent was used, the broad band at the beginning of the chromatogram was only slightly reduced; for this reason a more polar solvent (ACN) was then tested. When experiments with 2 ml of ACN as washing solvent were performed, it was demonstrated that not only was the broad band at the beginning of the chromatogram clearly reduced, but also that naproxen was selectively extracted from a mixture of NSAIDs in urine (Figure. 3). The recovery of naproxen was 60%, whereas fenoprofen, diclofenac and ibuprofen were completely stripped off the MIP. Benzoic acid was only slightly retained on the MIP but it could not be quantified because it appears at the beginning of the chromatogram with other interferent peaks. The decrease in recoveries observed for the NSAIDs in urine can be explained because although urine is mainly water (95%), other compounds can, in principle, interfere with the MISPE process. Nevertheless, we were able to selectively extract naproxen from the mixture of NSAIDs.

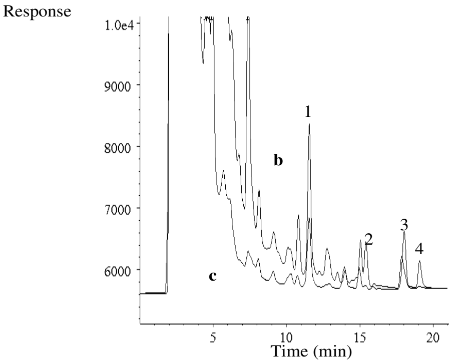
ACN was then used as the basis for further investigation.

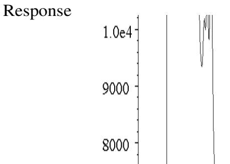
For comparative purposes experiments were also performed with a 200 mg commercial C_{18} Bakerbond (Deventer, Holland) SPE cartridge. When 25 ml of urine spiked with 55 µg l^{-1} of naproxen and 110 µg l^{-1} of the other NSAIDs was loaded, all compounds were retained on the cartridges and the recoveries were found to be nearly the same as those on the MIP. Figure 4 shows that after a clean-up step involving 2ml of ACN, all the compounds were completely eluted from the C18 sorbent, consistent with the fact that no selective interactions can be established between naproxen and this class of sorbent. This behaviour is completely different to that shown by the MIP in Fig. 3.

In order to decrease the time of analysis, the gradient profile was optimised, from 50 to 60% B in 10 min, to 100% B in 4 min and then isocratic elution for a further 2 min. Thus, after passing 25 ml of urine spiked with 55 µg l^{-1} of naproxen through the MIP and applying 2 ml of ACN for the clean-up step, naproxen was eluted after only 7 min (Figure 5). Under the optimised analysis conditions and with the shortened analysis time the linearity of the method was evaluated for naproxen. To check the linear range, 25 ml of urine, which did not contain any NSAIDs, was spiked with naproxen at concentrations between 110 and 3 µg l^{-1}.

Figure 3. Chromatograms obtained upon percolating 25 ml of a urine sample (pH 3) spiked at 55 µg l^{-1} with naproxen and 110 µg l^{-1} with a mixture of the other compounds through the MIP cartridge: a) blank of urine after a clean-up step with 2 ml of ACN, b) without a washing step, and c) with a washing step involving 2 ml of ACN: (1) naproxen, (2) fenoprofen, (3) diclofenac sodium, (4) ibuprofen.





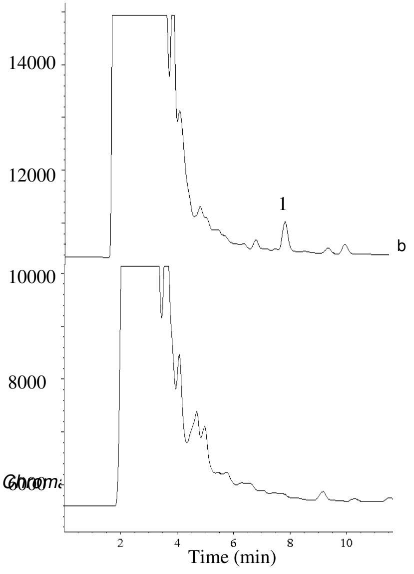


a

Figure 4. Chromatograms obtained upon percolating 25 ml of a urine sample (pH 3) spiked at 55 µg l^{-1} with naproxen and 110 µg l^{-1} with a mixture of the other compounds through a comme-rcial C_{18} solid-phase extraction cartridge: a) without a washing step, and b) with a washing step involving 2 ml of ACN. Peak designation as per Figure 3.

Figure 5. Chromatograms obtained upon decreasing the time of analysis. Pre-concentration of 25 ml of a urine sample (pH 3): a) without addition of naproxen, and b) spiked at 25 µg l^{-1} with naproxen and with a washing step involving 2 ml of ACN. Peak designation as per Figure 3

A washing step with 2ml of ACN was then applied. Good linearity was obtained with a determination coe-fficient (r^2) greater than 0.990. The repeatability for 25 ml of spiked (25 µg l^{-1} of naproxen) urine, expressed as R.S.D. (n = 3), was lower than 5%. The limit of detection of the method was established according to the signal to noise relation rule equal to 3 and was 3 µg l^{-1}. The application of the imprinted polymer to the MISPE of urine samples has therefore been successfully demonstrated. Significant-ly, not only can naproxen be selectively extracted from urine samples, but it can also be reliably and accurately quantified at low, bio-logically relevant levels (9–110 µg l^{-1}).



J. Chrom

CONCLUSIONS

This study shows, for the first time, the synthesis and the application in SPE of a polymer imprinted following a conventional non-covalent molecular imprinting protocol using (*S*)-naproxen as a template. The MIP was successfully applied as a selective sorbent in SPE, and it has been demonstrated that the MIP is able to selectively extract naproxen from human urine samples after a clean-up step involving 2 ml of ACN. Moreover, this work also demonstrates the feasibility of using a naproxen MIP, prepared using a straight-forward non-covalent synthetic procedure, for the direct determination of naproxen in urine. Due to the minimal sample preparation required and short time of analysis, this method appears to be very well-suited for the control of naproxen in human urine.

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2.4 SÍNTESI D'UN POLÍMER AMB EMPREMTA MOLECULAR PREPARAT AMB OXITETRACICLINA COM A MOLÈCULA *TEMPLATE* I APLICACIÓ A L'EXTRACCIÓ EN FASE SÒLIDA DE TETRACICLINES

Degut als bons resultats obtinguts en l'estudi anterior corresponent al MIP empremtat amb naproxen, es va decidir ampliar el camp d'aplicació i portar a terme l'extracció de compostos que es troben en mostres de matriu més complexa com poden ser les de teixit animal. Amb aquest objectiu, es va preparar un MIP amb oxtetraciclina (OTC) com a *template* i es va aplicar a l'extracció d'un grup d'antibiòtics (tetraciclines) en ronyó de porc. La presència d'aquests antibiòtics en mostres de teixit està regulada per la Unió Europea (UE), ja que si l'animal ha estat sacrificat abans de poder metabolitzar-los podrien passar fàcilment a la persona durant la ingesta d'aquest teixit i produir al·lèrgies en individus sensibles a aquests compostos. Per tal d'assegurar que els consumidors no estan exposats a concentracions de residus potencialment perilloses per la seva salut, la UE estableix el que es coneix com límits màxims de residu (MRL) [1]. La determinació d'aquests compostos és important i degut a la complexitat que aquest tipus de mostres presenten, l'aplicació dels MIPs com a sorbents pot resultar interessant.

La majoria de treballs on la MISPE ha estat aplicada a l'extracció de compostos de mostres biològiques tracten principalment amb biofluids [2-5], sent minoritaris els estudis realitzats en mostres de teixit [6-8]. Degut a la complexitat d'aquest tipus de mostra, normalment es treballa amb un sistema d'extracció en dues etapes on la mostra (l'extracte obtingut del tractament del teixit) es fa passar primer a través d'un sorbent comercial, per exemple C_{18}, Extrelut 20, etc. de manera que permet fer una primera neteja de la matriu de la mostra. A més a més, aquest mètode també presenta l'avantatge de poder preconcentrar els analits a través del MIP en absència d'aigua, ja que l'elució dels compostos retinguts al sorbent comercial es pot realitzar amb un solvent orgànic. No obstant, Muldoon *et al.* [6] és un dels pocs autors que va aplicar directament l'extracte de la mostra al MIP. Tot i no haver realitzat una etapa de neteja prèvia amb un solvent comercial, l'analit d'interès (atrazina) podia ser quantificat sense problemes d'interferents.

En el present estudi es presenta l'aplicació d'un MIP empremtat amb OTC tot i que també s'havia preparat un altre MIP sintetitzat amb la tetraciclina (TC) com a *template*. La síntesi dels dos MIPs amb aquestes molècules no va ser senzilla degut al gran nombre de grups funcionals que presenten a la seva estructura. Per tal de solubilitzar els *templates* i d'assegurar-nos que hi havia suficient monòmer funcional present al medi de síntesi per a que tots els grups funcionals de la TC i OTC poguessin establir interaccions específiques, la relació entre T:M:X emprada va ser 1:8:40. La diferent solubilitat entre els dos compostos va donar lloc a l'ús de l'acetonitril com a porogen en el cas de la TC i del dimetilsulfòxid (DMSO) en el cas de l'OTC.

D'igual manera que en els estudis anteriors, els MIPs sintetitzats es van aplicar com a sorbents en processos d'SPE. Primerament, es va intentar optimitzar el procés de MISPE en línia amb la cromatografia de líquids, però es van obtenir recuperacions molt baixes després de preconcentrar diversos patrons en aigua Milli-Q. Les baixes recuperacions podien ser degudes per una banda a que la quantitat de sorbent emprada no fos suficient i per una altra banda a que les condicions d'elució no fossin les adients. Per aquests dos motius els MIPs van ser aplicats finalment fora de línia.

El procés d'optimització es va dur a terme tant pel MIP empremtat amb la TC com amb l'OTC i es comprovar com en emprar una major quantitat de sorbent, els quatre antibiòtics estudiats (TC, OTC, 4-epiclorotetraciclina i doxiciclina) quedaven retinguts en ambdós MIPs. A més a més, també es van provar altres solvents d'elució en lloc de l'ACN/àcid acètic (99:1) corresponent al solvent orgànic de la fase mòbil i es va veure que en metanol amb un 10% de KOH 1M l'elució dels compostos era completa. Després d'optimitzar l'etapa de neteja, es va observar com tots dos MIPs presentaven reactivitat creuada. No obstant, degut a que el MIP empremtat amb l'OTC donava millors recuperacions per a tots quatre compostos, va ser aquest polímer el que es va emprar per a l'extracció d'aquests antibiòtics en extractes de teixit d'animal.

L'objectiu de l'estudi que s'adjunta a continuació va ser avaluar l'efecte que tenia sobre la selectivitat del MIP l'aplicació d'un extracte de teixit animal. Per aquest motiu i per tal d'evitar possibles pèrdues durant l'etapa de pretractament de mostra, es va fortificar amb la mescla de tetraciclines l'extracte final.

D’aquesta manera va ser possible avaluar les pèrdues degudes al procés de MISPE pròpiament.

L’extracte del teixit, que es va obtenir després d’un procés d’homogenització en un tampó EDTA-McIlvine [9] i de centrifugació, es va aplicar directament sobre el MIP, ja que durant l’etapa d’optimització de la MISPE es va comprovar com el MIP presentava bona afinitat pels compostos a extreure tot i la presència d’aigua a la mostra.

La recuperació per la TC i OTC va ser lleugerament inferior a l’obtinguda en aigua Milli-Q degut possiblement a que, tot i fer el tractament adient al teixit, restes de proteïnes i greixos són encara presents en l’extracte final, i per tant, poden donar lloc a l’obstrucció de les cavitats de reconeixement molecular, fent disminuir doncs la recuperació dels analits a extreure. Com a conseqüència de les restes de proteïnes a l’extracte final del teixit es va comprovar com un mateix cartutx no podia ser emprat per a més de dues extraccions.

Els resultats obtinguts en aquest estudi s’inclouen en el treball que s’adjunta a continuació i que ha estat enviat a la revista *Analytica Chimica Acta* per a la seva publicació.

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2.4.1 *Synthesis and application of an oxytetracycline imprinted polymer for the solid-phase extraction of tetracycline antibiotics*

SYNTHESIS AND APPLICATION OF AN OXYTETRACYCLINE IMPRINTED POLYMER FOR THE SOLID-PHASE EXTRACTION OF TETRACYCLINE ANTIBIOTICS

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Abstract

Two molecularly imprinted polymers (MIPs) were synthesised using tetracycline and oxytetracycline antibiotics as template molecules in non-covalent molecular imprinting procedures. After a chromatographic evaluation, the performance of the MIPs as selective SPE sorbents was evaluated. In this study, it was demonstrated that after a clean-up step to disrupt the non-specific interactions between the MIPs and the compounds retained on them, the polymers showed cross-reactivity for certain other tetracycline analytes. The feasibility of the MIP to selectively extract tetracycline antibiotics in pig kidney tissue extract was demonstrated when the oxytetracycline MIP, which gave rise to the best MISPE results, was then applied.

Keywords: Column liquid chromatography; Solid-phase extraction; Molecularly Imprinted Polymer; Tetracycline antibiotics; Tissue samples

INTRODUCTION

Molecularly imprinted polymers (MIPs) are tailor-made materials which can be applied in several different applications [1-3]. However, in the last few years they have been increasingly exploited as selective sorbents in molecularly imprinted solid-phase extraction (MISPE) [4]. To date, the main application of MISPE has been to the extraction of analytes from biological samples [5-14]. Biofluids such as plasma, serum and urine have been the most analysed biomatrices [5-10] and only few studies

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have been performed to the extraction of compounds from tissue samples [11-14].

Tissue samples are very complex matrices, for this reason, a pre-treatment step is always required. Then, the extract obtained is percolated through the MIP. Nevertheless, some proteins and lipids may still be present in the extract and subsequently they can hinder the access to the imprinted sites, which represents a decrease in the recognition properties of the MIP. Consequently, in most of the studies in which MISPE has been used for the extraction of analytes from tissue samples a two-step MISPE procedure has been applied [12-14]. Therefore, the extract coming from the pre-treatment sample is first percolated through a commercial sorbent ($C_{18,}$ OASIS HLB, Extrelut 20) and the retained analytes are then eluted and passed through the MIP.

Thus far, there is only a study in which the tissue extract has been applied directly through the MIP [11]. In this work, Muldoon *et al*. prepared a MIP with atrazine as template molecule and the polymer was then applied to selectively extract this analyte from beef liver extract which was obtained after a pre-treatment step. In this study the beef liver extract was directly applied to the MISPE cartridge and good results were obtained. Therefore, it was not necessary to use a commercial SPE sorbent before the MISPE cartridge.

The interest in detecting and quantifying drugs in animal tissues is increasing since it has been shown that these substances can leave residues in such tissues which can be directly toxic or cause allergic reactions in hypersensitive individuals. For this reason, in the present manuscript, the determination of tetracycline antibiotics has been studied. Two papers have been recently published regarding the imprinting with these compounds [15,16]. Nevertheless, in the study performed by Cai *et al* [15], the MIP prepared with TC was not used in a real sample application and, on the other hand, Suedee *et al.* [16] describes the application of molecularly imprinted membrane to the extraction of TC and its degradation products from water.

The present study describes for the first time the application of a MIP imprinted with tetracycline antibiotics to the direct extraction of tetracycline (TC) and oxytetracycline (OTC) from tissue sample extracts. TC and OTC were completely bound to their respective MIPs, which allow the tissue extract to be directly applied through the MIP.

EXPERIMENTAL

Reagents and standards

The chemicals used for the polymer syntheses were TC, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and dimethylsulfoxide (DMSO), all from Aldrich (Steinheim, Germany), OTC from Sigma (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium), and HPLC grade acetonitrile (ACN) from Rathburn Chemicals (Walkerburn, UK). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers, and the solvents dried over molecular sieves. The AIBN was recrystallised from acetone.

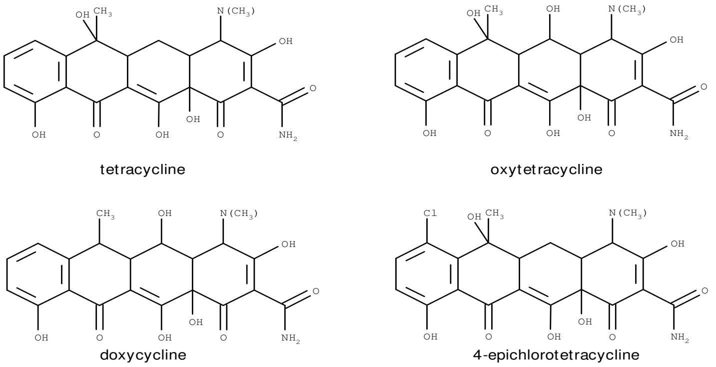
The HPLC-grade solvents (acetonitrile and methanol) were provided either by Rathburn Chemicals or SDS (Peypin, France) and the water collected from a Millipore water purification system (Milli-Q water). The acetic and hydrochloric acids were from Probus (Badalona, Spain) and dichloromethane (DCM) from SDS (Peypin, France).

The structurally related tetracycline compounds used to investigate the selectivity of the imprinted polymers were TC, OTC, 4-epichlorotetracycline hydrochloride (4-EPI), and doxycycline hydrochloride (DC). TC and OTC were kindly purchased by Cenavisa (Tarragona, Spain) and 4-EPI and DC were provided from Across Organics (New Jersey, USA). The structure of these compounds is shown in Figure 1.

Preparation of the Imprinted Polymers.

Polymers P1 and P2 were prepared by the non-covalent approach using TC and OTC as the template molecules, respectively. The same functional monomer (MAA) was used in both polymerisations but the porogenic solvents were different (for solubility reasons).

The pre-polymerisation mixture for P1 comprised TC (1.16 mmol), MAA (9.28 mmol), the cross-linking monomer EGDMA (46.41 mmol) and the initiator AIBN (1.02 mmol) dissolved in13.33 ml of the porogen acetonitrile, in a 25 ml thick-walled glass tube. This solution was cooled on an ice bath, sparged with oxygen-free nitrogen for five minutes, sealed under nitrogen and then left to polymerise in a water bath at 60 ºC for 24 h. The polymer monolith obtained was crushed, ground and wet-sieved using acetone to obtain polymer particles with diameters between 25 and 38 µm suitable for the chromatographic and MISPE evalua-tions.



tetracycline

oxytetracycline

doxycycline

4-epichlorotetracycline

Figure 1. Chemical structures of the tetracycline antibiotics used to study the selectivity of the MIPs.

The P2 pre-polymerisation mixture comprised OTC (1.16 mmol), MAA (9.28 mmol), EGDMA (46.42 mmol) and AIBN (1.02 mmol) dissolved in the porogen, DMSO, (13.33 ml) in a 25 ml thick-walled glass tube. The synthetic procedure followed during the polymerisation step was the same as for P1 polymer.

A reference, non-imprinted control polymer (NIP) based upon MAA (9.28 mmol) and EGDMA (46.41 mmol) with acetonitrile (13.33 ml) as the porogenic solvent, was prepared in absence of template.

Instrumentation

The polymers were initially evaluated in analytical columns to check the imprinting effects. 15 x 0,46 cm i.d. stainless steel HPLC columns were slurry packed with the ground polymer particles (25-38 μm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

The MISPE study was developed in an off-line mode using a solid-phase extraction manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump. 200 mg or 500 mg of each polymer suspended in MeOH was packed into an empty 6 ml polyethylene cartridge. The liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The loop for direct injection was 20 μl and the analytical column was a end-capped 25 x 0.4 cm i.d. Tracer Extrasil ODS2, 5 μm, supplied by Teknokroma.

Chromatographic Conditions

After packing of the polymers into analytical columns, the polymers were washed on-line with a mixture of acetonitrile/water/acetic acid (92.5/2.5/5 (*v/v/v*)) to eliminate interfering compounds arising from the synthesis (template and unreacted monomers).

For the chromatographic evaluation, 10 μl of each analyte and the void marker (acetone) were injected. P1 was evaluated using acetonitrile as the mobile phase at 0.21 ml min^{-1} in isocratic mode and P2 was evaluated using methanol as the mobile phase at 0.12 ml min^{-1} due to solubility issues and back-pressure problems. The NIP was evaluated under identical chromatographic conditions to P1 and to P2 in each case. The UV detector wavelength was set at 254 nm to detect acetone and 350 nm to detect the TCs. The analysis were performed at room temperature.

The parameters in the HPLC system for the MISPE experiments were as follows. The mobile phase was a mixture of two solvents: Milli-Q quality water containing 3% of MeOH and 1% of acetic acid (solvent A), and acetonitrile (solvent B). The flow-rate of the mobile phase was 1 ml min^{-1}, the gradient profile was 10-15% B from 0-15 min, 100% B at 35 min and then isocratic elution for 2 min. The column temperature was 35 ºC.

Molecularly Imprinted Solid-Phase Extraction Conditions

When tetracycline antibiotics were extracted from tissue sample extract, a standard protocol was followed in treating the matrix prior to the SPE [17]. Thus, 5 g of tissue was placed in a tube and 20 ml of EDTA-McIlvaine's buffer added. The mixture was homogenised for 1 min using an Ultra-Turrax T-25 (Jankle & Kunkel, IKA-Labortechnik, Staufen, Germany) and the mixture then centrifuged at 9,000 r.p.m. for 10 min. After this period, the pellet was blended and centrifuged twice more with 20 ml and 10 ml EDTA-McIlvaine's buffer, respectively. The supernatants obtained were mixed and centrifuged for 25 min and, after filtration of the supernatant, 25 ml of the filtrate was passed through the MISPE cartridge.

For the MISPE extractions, the cartridges were packed with 500 mg of polymer. 25 ml of tissue extracts (adjusted to pH 2 with HCl 0.1M) were spiked with the mixture of tetracycline antibiotics and applied to the cartridges which had already been conditioned with 10 ml Milli-Q water (pH 11 with NaOH 0.1M). The polymers were then washed with 3 ml of ACN and the retained analytes desorbed with 20 ml of MeOH containing 10% of 1 M aqueous KOH. 20 µl of each sample was injected on to the analytical column.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

MISPE

The SPE process was optimised for the TC and the OTC MIPs and the performance of these imprinted polymers for the extraction of tetracycline antibiotics was compared with the non-imprinted

In this study, the chromatographic evaluation of the MIPs was performed to have an idea about the imprinting effect of the polymers. For this reason, 10 µl of 10 mM of TC in ACN was injected onto the chromatographic columns containing P1 and the NIP and the capacity factors calculated (k'^{TC}_{P1}= 1.23, k'^{TC}_{NIP}= 0.31). From these results, it was concluded that P1 showed higher affinity for TC than the control polymer. Furthermore, there was extensive tailing of the TC peak on the P1 column, a characteristic of an imprinted stationary phase. The imprinting factor (IF= $k'^{TC}_{P1}/ k'^{TC}_{NIP}$) was also calculated (IF =4) and this was indicative of a good imprinted effect in P1.

Similar behaviour was observed when 10 µl of 10 mM of OTC in MeOH was injected onto the column packed with P2. The capacity factors were not calculated because the NIP was not prepared in the same conditions; nevertheless, the extensive tailing of OTC observed on P2 but not on the NIP, indicated a possible good imprinted effect.

The next step was to develop a SPE procedure to extract TC and OTC from animal tissue extracts. Both MIPs were applied to this end and in this way, it would be also possible to confirm the imprinting effect.

polymer. First of all, the conditioning and the loading steps were optimised. 10 ml of a standard solution, spiked with 5 mg l^{-1} of each compounds (TC, OTC, 4-EPI and DC), was passed through the cartridges packed, initially, with 200 mg of polymer. The sample prepared was in Milli-Q water at pH 2 and 9 and the cartridge conditioned with Milli-Q water at several pH values (2, 6 and 11). The analytes were only strongly retained on the MIPs when the conditioning was using Milli-Q water at pH 11 and the sample was loaded in Milli-Q water at pH 2 because in this way electrostatic interactions were established. When any other conditions were applied for either of these two steps, it was seen that the aqueous solution collected during the loading step contained approximately 70%-80% of the compounds indicating that minimum retention of the analytes had been achieved.

Using these efficient loading conditions, the elution step was then optimised. When ACN or MeOH were used, the recoveries of the analytes were lower than 50%. However, when 3% of 1 M aqueous KOH was added to the MeOH, the recoveries increased significantly. This is likely to be because the interactions between the tetracyclines and the MIPs are disrupted at this pH. When volume of the elution solvent was studied, it was demonstrated that any volume lower than 10 ml was not sufficient to elute the TCs completely from the polymers. For this reason, 10 ml of this solvent was finally chosen as the optimum volume.

It is well known that compounds can be retained on MIPs due to both specific and non-specific interactions. Thus, it was thought that a suitable clean-up process would remove the non-specifically bound compounds from the MIPs, while the analytes used as templates would remain bound. Several organic solvents such as DCM, ethyl acetate and ACN, were investigated as washing solvents. A drying step which consisted of applying a vacuum to the SPE cartridges for 15 min prior to the clean-up was included to remove small amounts of water remaining on the cartridge [18-20]. When DCM or ethyl acetate were used, the non-specific interactions established between the analytes and P1, P2 and NIP were not removed, thus all the compounds present in the mixture previously described (TC, OTC, 4-EPI and DC) were still retained on the polymers. Nevertheless, a selective clean-up step was possible with ACN. Different volumes of this solvent were tested and the imprinting effect was revealed when 3 ml was applied, as can be seen in Figure 2. In this situation, tetracycline antibiotics were retained on both MIPs, but not in the NIP where the analytes were nearly washed off during this clean-up step. Figure 2 also shows that in these clean-up conditions, the analytes are stronger retained on OTC MIP than in TC MIP which represents higher recoveries for tetracycline

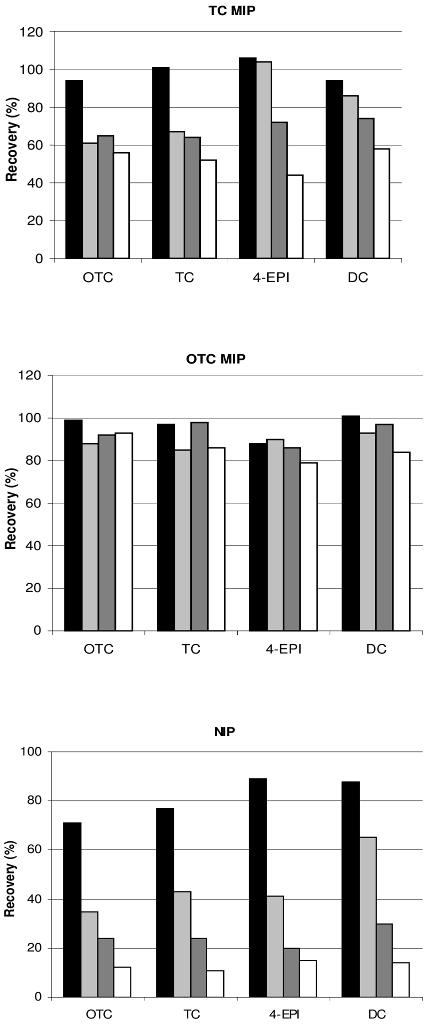


Figure 2. Recovery of the molecularly imprinted polymers when 10 ml of a standard solution spiked at 5 mg l^{-1} with the mixture of compounds was percolated through each polymer. a) TC MIP (P1), b) OTC MIP (P2), and c) NIP, without clean-up (■) and when a clean-up step was applied using 1 (■), 2 (■) and 3 (□) ml of ACN, respectively, as the washing solvent. (RSDs= 11% (n= 3))

antibiotics in OTC MIP. This allowed the conclusion to be drawn that OTC MIP shows more cross-reactivity [21] for other structurally close analogues than TC MIP.

In order to decrease the cross-reactivity shown by the MIPs, acid acetic was added to the ACN washing solvent (ACN-acetic acid (99:1)). This mixture was then evaluated as a potential solvent for the clean-up step, but there was no improvement in terms of selectivity, neither for TC MIP nor OTC MIP, because all the analytes in the sample were still retained (recoveries slightly lower than those using ACN alone). This allows to confirm that the interactions established between the analytes and the MIP during the loading step are based on selective ionic interactions since they are not disrupted by the presence of acetic acid in this clean-up step. ACN was finally chosen as the selective washing solvent for further experiments.

The recovery of the compounds at different sample volumes (10, 25, 50 and 100 ml) was studied. When the sample volume was increased to 25 ml it was observed that the recoveries for the tetracyclines decreased slighlty; consequently, the cartridges were packed with 500 mg of polymer, which allowed the retention of the analytes to be improved. The volume of MeOH and the percentage of KOH used in the elution step had to be increased to 20 ml and 10%, respectively, to elute the retained analytes. At these conditions, after performing the clean-up step the recoveries were similar than those obtained using the 200 mg cartridge.

It has been shown that after the clean-up step, the OTC MIP gives not only the best recovery values, but also shows a higher imprinting effect than the TC MIP in the MISPE experiments. For this reason, the OTC MIP was selected for further applications using tissue samples extracts.

MISPE of Tissue Sample Extracts

Tissue samples are complex biological matrices and for this reason an exhaustive pretreatment of the sample is always required. The use of a selective sorbent such a MIP can be very useful to obtain cleaner chromatograms. However, if proteins or lipids remain in the resultant tissue extract they can hinder the access to the imprinted sites and the recoveries can decrease. To evaluate the effect of a tissue extract in the procedure of molecular recognition, the extract was spiked with a mixture of the tetracycline antibiotics. In this way, it was possible to check the recovery and selectivity of only MISPE process avoiding possible looses of recoveries arising from the tissue pre-treatment step.

5 g of a pig kidney tissue was selected and the standard sample treatment [22] followed prior to the MISPE experiments. After performing the standard sample treatment, 25 ml of the extract obtained was spiked with 0.12 mg l^{-1} of TC and OTC (600 µg of TC and OTC per kg of tissue) to evaluate the performance of the MIP in this kind of matrix. The tissue extract was directly applied to the MIP since it has been demonstrated that TC and OTC are completely retained on OTC MIP when they are applied in water samples. After the loading step, the cartridge was dried and the polymer was then washed with 3 ml of ACN and the compounds eluted with 20 ml of MeOH containing 10% of 1 M aqueous KOH. The eluate was evaporated to dryness and the residue reconstituted in 1 ml of HCl (0.01 M). 20 µl of this extract were injected onto the analytical HPLC column.

No peaks were observed at the retention time of the analytes when the non-spiked blank kidney tissue was analysed. When the tissue extract was spiked with TCs, the recoveries in the absence of the clean-up step were 66% and 69% for OTC and TC respectively, which were lower than those obtained with the acidified water standard solution. The lower recoveries obtained for the tetracycline antibiotics studied could be explained as a result of the matrix content still present in the solution extract after the standard sample treatment. When the clean-up step was included, the recoveries obtained were the same as those without washing the cartridge. Figure 3a shows the chromatogram obtained when the clean-up step was not performed and it should be pointed out that the impurity which coelutes with the TC analyte is nearly completely removed after the clean-up step as can be observed in Figure 3b, which represents a better quantification for the TC analyte. The repeatability of the method for 25 ml of spiked (0.12 mg ml^{-1}) tissue sample extract, expressed as RSD (n=3), was lower than 8%. Thus, the selectivity and the recovery of MISPE when biological extracts are percolated through P2 have been checked and the applicability of the MIP demonstrated.

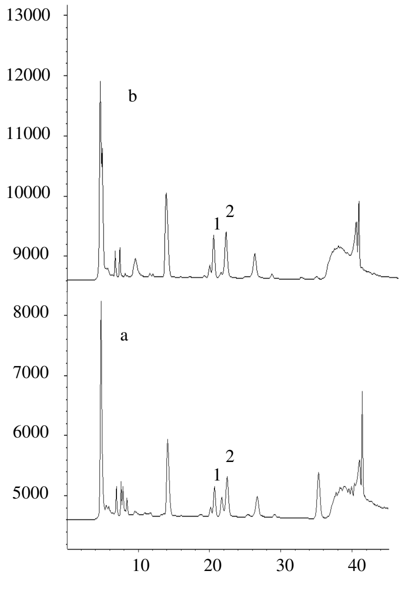


Figure 3. Chromatograms obtained by MISPE with the OTC imprinted polymer (P2) using 25 ml of extract from the standard tissue sample treatment (pH 2) spiked at 0.12 mg ml^{-1} with the mixture of compounds. a) Without clean-up step, and b) With a

clean-up step involving 3 ml of ACN. Peak designation: **1**= OTC, **2**= TC.

CONCLUSIONS

In this paper, two imprinted polymers (TC MIP and OTC MIP) prepared by a non-covalent molecular imprinting protocol, have been synthesised using TC as template molecule for TC MIP and OTC for OTC MIP. Both MIPs were applied in SPE protocols as selective sorbents. The imprinting effect and selectivity of the MIPs was confirmed and it was seen that OTC MIP was deemed to be the most promising polymer for further study because it showed the greatest imprinting effect and the highest recoveries for all the compounds pre-concentrated. Thus, only the OTC MIP was applied to the direct extraction of TC and OTC from tissue extracts. It has been demonstrated the applicability of the MIP when complex biological samples are passed through it.

Acknowledgements

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2.5 POLÍMERS AMB EMPREMTA MOLECULAR PER A L'EXTRACCIÓ SELECTIVA DE FLUOROQUINOLONES EN MOSTRES BIOLÒGIQUES

Degut a que són minoritàries les aplicacions de la MISPE a l'extracció de compostos en teixits animals i als resultats obtinguts en l'estudi anterior, en aquest apartat es presenta la síntesi de tres nous MIPs, dos d'ells empremtats amb enrofloxacina (ENRO) i aplicats a l'extracció d'aquest compost en orina i en teixit d'animal, i un tercer MIP preparat amb ciprofloxacina (CIPRO) com a *template* i aplicat a l'extracció d'aquest mateix compost en mostres d'orina.

L'ENRO i la CIPRO formen part d'una família d'antibiòtics coneguda com fluoroquinolones i són àmpliament utilitzades per al tractament d'infeccions, principalment urinàries, intestinals i biliars, tant en medicina humana com en veterinària, i en el cas dels animals també són emprades per potenciar el seu creixement. Així doncs, igual que les tetraciclines, la presència de fluroquinolones en teixit animal està regulada per la legislació europea. Degut a la complexitat que presenten aquests tipus de mostres l'ús de MIPs com a sorbents pot resultar interessant per a eliminar amb més facilitat la matriu de la mostra i així poder quantificar els compostos als nivells exigits per la normativa, que en el cas de la ENRO, el Consell Regulador 2377/90 [1] estableix els MRLs per aquest compost com la suma de la ENRO i el seu metabòlit CIPRO (Taula 2.5.1).

La síntesi dels MIPs empremtat amb l'ENRO es va portar a terme en dos solvents diferents: diclorometà i cloroform, degut a que l'ENRO era soluble en tots dos i així estudiar l'efecte del porogen en la selectivitat del MIP. D'aquesta manera es va poder triar finalment el MIP que resultava més adient per a l'aplicació a portar a terme.

Inicialment, tots dos MIPs es van avaluar cromatogràficament. El perfil d'elució observat per a l'ENRO en cadascun dels MIPs era l'esperat per a qualsevol polímer empremtat: pics amb llargues cues i temps de retenció elevats. Dels factors d'empremta molecular que es mostren a la Taula 2.5.2 es pot deduir com, efectivament, els dos polímers estaven empremtats i dels valors de RI es dedueix com el MIP preparat en DCM té una reactivitat creuada per la CIPRO molt més marcada. Per tant, sembla evident que el porogen juga un paper important en les característiques finals de cada MIP.

Taula 2.5.1 MRLs per a la enrofloxacina en diversos teixits d'animals destinats al consum humà

Substància farmacològicament activa	**Residu**	**Espècie Animal**	**MRL (µg kg^{-1})**	**Teixit**
Enrofloxacina	Enrofloxacina + Ciprofloxacina	Pollastre	100	Múscul
		Boví	100	Múscul
			100	Greix
			300	Fetge
			200	Ronyó
			100	llet
		Porcí	100	Múscul
			100	Pell+greix
			200	Fetge
			300	Ronyó
		Pollastre	400	Múscul
			250	Pell+greix
			800	Fetge
			1000	Ronyó

Taula 2.5.2 Valors dels Factors de capacitat (K'), dels Factors d'empremta molecular (IF) i de l'Índex de Retenció normalitzat (RI) per a cadascun dels MIPs sintetitzats amb l'ENRO com a *template* i amb diclorometà i cloroform com a porògens.

	DCM				cloroform			
Compost	k'$_{MIP}$	k'$_{NIP}$	IF	RI	k'$_{MIP}$	k'$_{NIP}$	IF	RI
Enrofloxacina	3.9	0.2	17.4	1.0	2.2	1.1	2.2	1.0
Ciprofloxacina	6.0	0.1	75.3	4.3	13.4	4.7	2.8	1.3

Tots dos MIPs es van provar com a sorbents en SPE. El procés d'optimització de la MISPE es va dur a terme tant amb una mostra d'aigua Milli-Q com amb metanol que contenien una barreja de tres fluoroquinolones (norfloxacina, CIPRO i ENRO) i es va comprovar com l'efecte d'empremta molecular era màxim quan la mostra s'aplicava en en metanol. Per aquest motiu es va desenvolupar un sistema d'extracció en dues etapes; primer la mostra es preconcentrava a través d'un cartutx comercial (OASIS HLB) i l'extracte recollit en metanol de l'etapa d'elució es transferia cap al MIP. D'aquesta manera els analits passaven a través del MIP en el solvent on el MIP presentava un major efecte d'empremta molecular. Per una altra banda, cal remarcar que aquest sistema d'extracció permetrà eliminar millor les restes de matèria orgànica que puguin quedar a la mostra d'orina després de filtrar-la i a la mostra de teixit després de triturar-la, homogenitzar-la i centrifugar-la i, per tant, allargar la vida útil del cartutx. En altres estudis en què s'analitzen teixits [2,3], s'ha comprovat com l'etapa d'extracció també es realitza mitjançant l'ús de dos cartutxos.

En aplicar 25 ml d'un blanc d'orina a través d'aquest doble sistema d'extracció, es va poder comprovar com el MIP preparat en cloroform presentava més selectivitat, ja que tal i com s'observa a la Figura 2.5.1 el cromatograma corresponent a aquest MIP era molt més net.

Tot i així es va continuar treballant amb els dos polímers i en preconcentrar una mostra d'orina fortificada amb una mescla de CIPRO i ENRO es va comprovar com el MIP preparat en DCM mostrava una reactivitat creuada per la CIPRO molt més gran que el MIP preparat en cloroform. Aquesta diferència era d'esperar degut als resultats obtinguts en l'avaluació cromatogràfica. Per aquest motiu es va decidir treballar amb el MIP preparat en DCM, ja que l'objectiu de l'estudi era extreure l'ENRO de mostres de teixit animal i degut que la normativa europea estableix els MRLs per a l'ENRO com la suma d'ENRO i CIPRO, aquest MIP resultava idoni.

El MIP preparat en DCM es va aplicar finalment per a l'extracció d'ENRO i CIPRO en fetge de porc. En aquest cas, no va ser necessari introduir una etapa de neteja abans de l'elució dels analits del MIP, no només perquè l'extracte obtingut ja era prou net, sinó perquè a més a més, d'aquesta manera s'evitaven possibles pèrdues de la CIPRO durant l'etapa de neteja. D'aquesta manera es poden quantificar les dues fluoroquinolones tal i com estableix la normativa europea [1].

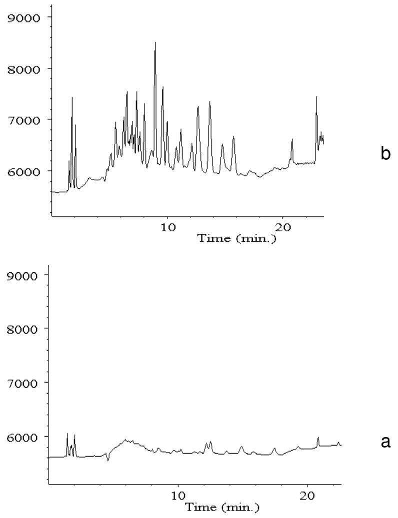

Figura 2.5.1 Cromatogrames obtinguts després de preconcentrar 25 ml d'un blanc d'orina a través del cartutx d'OASIS i del MIP preparat en cloroform (a) i del MIP preparat en DCM (b).

El tercer MIP sintetitzat amb una fluoroquinolona es va preparar amb la CIPRO com a *template* i emprant DCM com a porogen degut a que en cloroform aquest compost era menys soluble. La síntesi es va dur a terme en les mateixes condicions que el MIP empremtat amb la ENRO en DCM i es va aplicar a l'SPE d'un grup de fluoroquinolones.

L'avaluació cromatogràfica d'aquest MIP va permetre comprovar com a diferència del seu homòleg preparat amb la ENRO com a *template*, la reactivitat creuada que presentava aquest nou MIP era molt inferior.

Aquest MIP també es va aplicar com a sorbent en processos d'extracció en fase sòlida i, igual que en els dos MIPs empremtats amb ENRO, es va observar que quan la mostra s'aplicava en MeOH, l'efecte d'empremta molecular es veia afavorit. Per tant, quan es va dur a terme l'extracció de la CIPRO de mostres d'orina va ser necessari aplicar el procés d'extracció en dues etapes descrit prèviament. D'aquesta manera no només era possible fer un canvi del solvent de la mostra sinó que el procés de MISPE era tan eficient que després d'una etapa de neteja amb un solvent orgànic polar i apròtic, dimetilformamida, s'obtenien cromatogrames molt nets i els extractes de les mostres d'orina es van poder injectar directament a un espectròmetre de masses (MS). La selectivitat del MIP junt amb la selectivitat i sensibilitat del MS, va permetre la quantificació i confirmació de la CIPRO a baixos nivells i reduir el temps d'anàlisi en eliminar la columna cromatogràfica.

Tant el primer dels estudis descrits, on el MIP s'ha preparat amb ENRO com a *template,* com l'estudi dut a terme amb la CIPRO com a *template* han estat enviats a la revista *Journal of Chormatography A* per a la seva publicació.

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2.5.1 *Novel enrofloxacin imprinted polymer applied to the solid-phase extraction of fluorinated compounds from urine and tissue samples*

NOVEL ENROFLOXACIN IMPRINTED POLYMER APPLIED TO THE SOLID-PHASE EXTRACTION OF FLUORINATED QUINOLONES FROM URINE AND TISSUE SAMPLES

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Abstract

A new molecularly imprinted polymer, prepared following a non-covalent approach, was synthesised using enrofloxacin as a template molecule. The imprinting effect of the polymer was verified by chromatographic evaluation and, interestingly, this evaluation also revealed that the imprinted polymer showed a high degree of cross-reactivity for ciprofloxacin, the major metabolite of enrofloxacin. The molecularly imprinted polymer was then applied as a selective sorbent in a two-step, solid-phase extraction method focussing upon complex biological matrices, specifically urine and pig liver. This two-step solid-phase extraction protocol, in which a commercial OASIS HLB cartridge and a molecularly imprinted solid-phase extraction (MISPE) cartridge were combined, allowed enrofloxacin and ciprofloxacin to be determined by liquid chromatography coupled to a UV detector at levels below the maximum residue limits established by the European Union.

Keywords: Molecularly imprinted polymer; Solid-phase extraction; Enrofloxacin; Human urine; Pig liver

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INTRODUCTION

Quinolones are antibiotic widely to prevent and treat a large variety of infectious diseases in human and veterinary medicine [1]. In veterinary medicine quinolones also promote animal growth. It is well known that residues of such antibiotics may persist in edible animal tissues, which makes quinolones potentially hazardous to human health.

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To ensure that consumers are not exposed to quinolone residues at potentially harmful concentrations, the European Union (EU) has established maximum residue limits (MRLs) for such compounds present in edible tissues [2]. Thus, it is important to develop analytical methods for determining these drugs present in biomatrices at low levels. Most of the analytical methods established thus far for quinolones are based on liquid chromatography (LC) [3-6] and, given the chemically complex nature of sample matrices such as biological fluids and animal tissues, this makes a sample preparation step necessary prior their analysis.

Over the last few years, solid-phase extraction (SPE) has become the most commonly used sample preparation technique, not only for cleaning of the sample matrix but also to enrich the analytes present prior to analysis. Nevertheless, due to the lack of selectivity of the commercial SPE sorbents, other materials with high selectivity, such as immunosorbents (IS) and molecularly imprinted polymers (MIPs) [7], have been developed and applied to extraction procedures. The expensive and time-consuming proce-dure for the production and isolation of antibodies as well as their lack of availability, have led to MIPs being exploited widely in molecularly im-printed solid phase extraction (MISPE) [8].

MISPE has been applied successfully to the extraction of drugs from several different biological fluids, including urine, serum and plasma [9-15], but there are relatively few studies focussing upon tissue samples [16-17]. In these two papers, two-step SPE processes were developed to extract clenbuterol from calf and bovine liver, respectively. Brambilla *et al* [16] used an Extrelut 20 column to extract clenbuterol in the first instance; the eluate collected was then applied to a MIP. In contrast, Crescenzi *et al* [17] used a matrix solid-phase dispersion pre-treatment to prepare the samples, a technique that greatly simplifies the pre-treatment of solid samples, but an additional clean-up step was, nevertheless, still required. For this reason, Crescenzi et al. combined MSPD with MISPE.

In the present paper, we describe the first example of a fluorinated quinolone, enrofloxacin, used as template to prepare a MIP which is applied as a selective sorbent, to extract ENRO via a two-step MISPE procedure from human urine but also to extract ENRO and ciprofloxacin (another fluorinated quinolone) from pig liver. This SPE protocol allowed matrix compounds to be nearly virtually removed from the sample and ENRO to be quantified at MRL levels by a liquid chromatographic system (HPLC) coupled to a UV detector.

EXPERIMENTAL

Reagents and standards

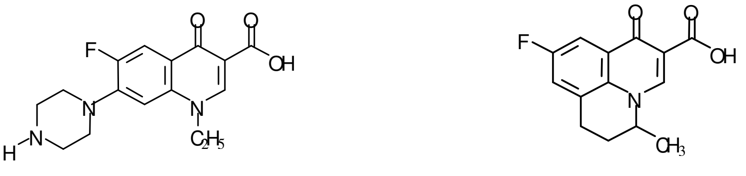
The reagents for the polymer synthesis were as follows: enrofloxacin (ENRO), which was kindly donated by Cenavisa (Reus, Spain), methacryclic acid (MAA) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2’-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium), and dichloromethane (DCM) from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers, and the solvent dried over 4 Å molecular sieves. The AIBN was recrystallised from acetone.

HPLC-grade solvents used to prepare the LC mobile phases were acetonitrile (ACN), provided by SDS (Peypin, France), and water, which was collected from a Millipore water purification system (Milli-Q water). Phosphoric acid and sodium dihydrogen phosphate, which were from Probus (Badalona, Spain) and Panreac (Barcelona, Spain), respectively, were used also to prepare the mobile phases. Other pH modifiers used were acetic acid and hydrochloric acids from Probus, formic acid from Merck (Darmstad, Germany) sodium hydroxide (NaOH) from Prolabo (Fontenay, France) and triethylamine (TEA) from Aldrich.

Some fluorinated quinolones such as norfloxacin (NOR) and ciprofloxacin (CIPRO) and the quinolone flumequin (FLU), all them related structurally to ENRO (Figure 1), were used to investigate the cross-reactivity of the MIP. NOR and FLU were supplied by Sigma (Steinheim, Germany) and CIPRO was kindly donated by Cenavisa (Tarragona, Spain). Standard solutions for each compound were prepared monthly at a concentration of 100 mg l^{-1} in 0.1 M NaOH.

Several tetracycline compounds, including tetracycline (TC), oxy-tetracycline (OTC), and doxycycline (DC), were also used in certain experiments to probe the selectivity of the MIP for other group of antibiotics. The tetracyclines were all purchased from Sigma.

ENROFLOXACIN (ENRO) CIPROFLOXACIN (CIPRO)



NORFLOXACIN (NOR) FLUMEQUIN (FLU)

Figure 1. Chemical structures of the fluorinated quinolones studied.

Preparation of the Imprinted Polymer.

For the preparation of the enrofloxacin imprinted polymer, the template (ENRO, 0.21g, 0.58 mmol) was dissolved in the porogen (DCM, 6.66 ml) in a 25 ml thick-walled glass tube. The functional monomer (MAA, 0.40g, 4.64 mmol), the cross-linking monomer (EGDMA, 4.60g, 23.20 mmol) and the initiator (AIBN, 0.08g, 0.51 mmol) were then added. The resultant solution was cooled on an ice bath and degassed with oxygen-free nitrogen for five minutes before being sealed under nitrogen. The polymerisation was allowed to proceed at 58ºC for 24h in a water bath. After this period, the glass tube was broken and the monolith obtained was ground mechanically and wet sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 µm suitable for the MISPE evaluations.

A non-imprinted polymer (NIP) was prepared and treated in an identical manner to the MIP, the only difference being that there was no ENRO present during polymerisation

Instrumentation

High performance liquid chromato-graphy (HPLC) was the primary tool used for the evaluation of the imprinting effect. In this regard, stainless steel HPLC columns (15 x 0.46 cm i.d.) were slurry packed with the ground polymer (MIP or NIP) particles (25-38 µm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an automatic injector and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in the pre-screening work.

For the two-step MISPE of biological samples, a commercial SPE sorbent, such as OASIS HLB (3 ml, 60 mg) (Milford, MA, USA), was used in the first step. The eluate collected from the commercial sorbent was then applied to a 6 ml polyethylene SPE cartridge which contained 200 mg of the MIP. All the cartridges were connected to a manifold, supplied by Teknokroma (Barcelona, Spain), coupled to a vacuum pump.

For the analytical method, the liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The injection volume was 20 µl and the analytical column was a 15 x 0.46 cm i.d. Eclipse XDB-C8, 5 µm, supplied by Hewlett Packard (Palo Alto, USA).

Chromatographic Conditions

Prior to the chromatographic evaluation of the MIP and the NIP, the columns were washed on-line with a mixture of chloroform/acetic acid (95:5 *(v/v)*) to remove the template and monomers arising from the synthesis. Thereafter, the columns were washed with a chloroform/acetic acid (99:1) mobile phase until a stable baseline was observed. 20 µl of 10 mM enrofloxacin in chloroform/acetic acid (99:1) and 1 µl of the void marker (acetone) were then injected. The NIP was evaluated under identical chromatographic condi-tions. The analysis was performed in isocratic mode at 1 m min^{-1} and the UV detector wavelength was set at 280 nm. The analysis was performed at ambient temperature.

The chromatographic conditions for the MISPE experiments were as follows. The mobile phase was a mixture of two solvents, ACN (solvent A) and an aqueous phase (solvent B) which contained 0.02 M of sodium dihydrogen phosphate and 2 ml of TEA. The pH of the aqueous phase was adjusted to 3.0 with phosphoric acid and filtered through a 0.22 µm filter. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was from 5 to 10% B in 5 min, isocratic elution for 6 min and the B content was increased to 20% in 5 min, to 50% B in 2 min and then isocratic elution for 2 min, i.e., the overall run time was 25 min. The column temperature was 35 ºC and all compounds were detected at 280 nm, except for FLU which was detected at 324 nm.

MISPE Conditions

For the two-step MISPE experiments, urine and tissue extracts (adjusted to pH 3) were passed through the OASIS cartridge conditioned previously with 5 ml of MeOH and 2 ml of Milli-Q water. After sample application, a washing step with 2 ml of Milli-Q water was applied and the analytes then desorbed using 10 ml of MeOH. This methanolic solution containing all the compounds from the commercial cartridge was then passed through the MIP previously conditioned with 6 ml of MeOH. The MIP was washed with 6 ml of ACN/acetic acid (95:5) only when urine samples were analysed. For the elution step 3 ml of ACN/(4%) formic acid (1:4) were used and 20 µl of each sample was then injected onto the analytical column.

Analysis of biological samples

The urine samples were collected from a healthy volunteer. Urine was store in a freezer at -20ºC until use and filtered through a 0.22 µm syringe filter before being applied to the OASIS and MIP cartridges.

For the extraction of quinolones from pig liver, a standard protocol was followed to prepare the matrix prior to the SPE [18]. To 5 g of tissue 5 ml of 0.1 M HCl was added. The mixture was homogenised for 1 min using an Ultra-Turrax T-25 (Jankle & Kunkel, IKA-Labortechnik, Staufen, Germany) and the mixture then centrifuged at 9,000 r.p.m. for 20 minutes. After this period, the pellet was blended and centrifuged again with 5 ml of 0.1 M HCl. The supernatants obtained were combined and centrifuged for 15 min and, after filtration of the supernatant through a 0.45 µm syringe filter, the filtrate was passed through the OASIS and MIP cartridges.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

Chromatographic evaluation was used as the first tool to investigate the imprinting effect of the MIP. Chloroform was chosen as the mobile phase, but due to the problems associated with the application of MIPs as stationary phases in HPLC (very broad, tailing peaks and time-consuming analyses) a modifier, such as acetic acid, was added to the mobile phase. In this way, the MIP and the modifier compete for rebinding to the analyte, which represents an improvement of the peak symmetry and reduces simultaneously the analysis time. Chloroform/acetic acid (99:1) was then used as an optimal mobile phase in the present. From the retention times of ENRO on the MIP and the NIP columns, the capacity factors and the imprinting factor were calculated (Table 1). CIPRO was also injected onto the MIP and the NIP columns to probe the selectivity of the MIP for other structurally related analytes. The capacity factors and the imprinting factor for CIPRO were also calculated in a similar fashion to those for ENRO and are also presented in Table 1.

Table 1. Capacity factors and imprinting factors arising from the chromatographic evaluation of the enrofloxacin imprinted polymer.

Compound	**k'^{a}_{MIP}**	**k'^{a}_{NIP}**	**IF^{b}**	**RI^{c}**
Enrofloxacin	3.90	0.22	17.38	1.00
Ciprofloxacin	6.02	0.08	75.30	4.33

[a] k' (Capacity Factor) [b] IF (Imprinting Factor)
[c] RI (Normalised Retention Index)

The Normalised Retention Index (RI) was calculated to enable the k' values of ENRO and CIPRO to be compared [19]. As can be seen in Table 1, the MIP shows high affinity for the template, ENRO, as would be expected for an imprinted material, but also higher affinity for CIPRO than ENRO. The latter observation can be rationalised by considering the chemical structures of ENRO and CIPRO, as shown in Figure 1. The only difference between the two structures arises in the piperizinyl ring. In terms of their relative ability to form an electrostatic interaction with carboxylic acid, in the absence of steric effects one would expect the interaction with ENRO (tertiary amine) to be stronger than that with CIPRO (secondary amine). Given that the expected order of elution was reversed, we can only assume that steric effects are playing a role in the binding.

The selectivity and retention of analytes on the MIP compared to the control polymer has thus been verified. Moreover, it has also been demons-trated that the MIP shows a high degree of cross-reactivity for CIPRO. The chromatographic evaluation has provided useful information about the MIP for the subsequent development of the MISPE method.

MISPE

Several parameters must be optimised in MISPE experiments in order to maximise the selective recognition of the analyte. In order to demonstrate the selectivity of the MIP for the ENRO, other structurally related compounds such as NOR, CIPRO and FLU were also added to the sample. The conditioning and the loading steps were optimised first of all. Thus, 10 ml of a mixture containing 0.3 mg l^{-1} of NOR, CIPRO, ENRO and FLU was prepared in two solvents, methanol (MeOH) and Milli-Q water at different pH values (3, 7, 11) and passed through the MIP. The cartridge had been conditioned previously with 6 ml of the same solvent in each case.

When the sample was applied in MeOH, all the analytes were retained on the MIP mainly by selective interactions, except for FLU which was not retained at all during the loading step. This may well be because FLU lacks a piperizinyl ring, believed to be one of the key structural features necessary for binding to the MIP.

For the elution step several solvents were investigated. The best conditions found to quantitatively elute and recover all the retained analytes was to use 3 ml of ACN/formic acid (4%) (1:4).

When an identical mixture of quinolones was prepared in Milli-Q water at pH 3, all the analytes were completely retained, even FLU, although it was only recovered in 60%. In this situation (aqueous environment) hydrophobic (non-specific) interactions dominate the retention process. When other pH values were investigated (pH 7 and pH 11), the compounds were completely retained at neutral pH but not at basic pH (recoveries from 47-69% for all compounds). Retained analytes were eluted using the same conditions as described previously.

In order to enhance the selectivity of the MIP and decrease the cross-reactivity, a clean-up step (selective washing step) was included in the MISPE procedure. This step was first optimised when the sample was applied in MeOH. Thus, DCM, toluene, chloroform and ethyl acetate were investigated as potential washing solvents for the clean-up step, but the cross-reactivity of the MIP was not decreased. The same behaviour was observed when the effect of more polar solvents, such as ACN and dimethylformamide (DMF), was also investigated. It should be pointed out that these experiments were also performed in the NIP and a clean-up with 4 ml of DMF was enough to completely remove the fluorinated quinolones. The strong cross-reactivity shown by the MIP was expected because of the previous results obtained from the chromatographic evaluation. Therefore, acetic acid was added to the previously described solvents in order to decrease the cross-reactivity since this modifier competes with the binding sites for rebinding the analytes. The best results were obtained using 6 ml of acetic acid in ACN (5:95). Under these conditions there was an improvement since NOR and CIPRO were recovered at a level of 40% whereas ENRO remained completely bound to the MIP. ACN/acetic acid was used as the wash solvent in all subsequent experiments.

The clean-up step was also studied when the sample was applied to the cartridge in acidified Milli-Q water (pH 3). After drying of the cartridge for 15

min, the effect of using the same set of wash solvents was investigated. In this situation, when ACN or DMF were used all the compounds were close to being completely removed from both the MIP and NIP, even although only 2 ml of solvent was used. For this reason, in all subsequent experiments the analytes were applied from MeOH.

A mixture of tetracyclines (TC, OTC and DC) in MeOH was percolated through the MIP together with the quinolones to explore the selectivity of the MIP for another group of antibiotics. Tetracyclines are also used extensively to treat animal and human diseases. When 10 ml (0.3 mg l^{-1}) of a mixture of these tetracyclines and fluorinated quinolones in MeOH was applied to the MIP cartridge, it was found that the tetracyclines were only retained at a level of 40%, presumably because they were not able to establish specific interactions with the MIP. A subsequent clean-up step involving ACN/acetic acid (95:5) eliminated the tetracyclines from the MIP, leaving only the quinolones remained still bound to the MIP (Figure 2).

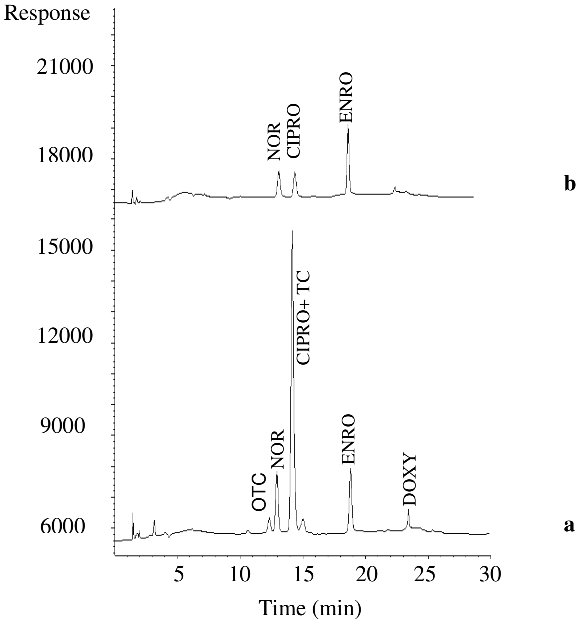


Figure 2. Chromatograms obtained from the percolation through the MIP of 10 ml of sample prepared in MeOH containing 0.3 mg l^{-1} of a mixture of several quinolones and tetracyclines. A) When a clean-up step was not performed; b) with a clean-up step using 6 ml of ACN/acetic acid (95:5).

It has been demonstrated that the MIP was imprinted. However, to make best use of the selective interactions between the analytes and the MIP the sample must be applied in MeOH. For this reason, a two-step SPE method was developed which allowed us to not only apply the biological samples to the MIP from a methanolic solution, but also to obtain cleaner extracts.

Analysis of Biological Samples

To demonstrate the value of the MIP as a sorbent for sample pre-treatment, two different real samples, such as human urine and pig liver, were investigated. These complex matrices make the determination of quinolones difficult at low concentrations, especially when the analytes have to be extracted from tissue samples and quantified at MRL levels. In order to completely suppress matrix interferences, a two-step SPE procedure suitable for monitoring quinolones in urine and pig liver samples was developed. In the two-step MISPE method used to extract quinolones from biological samples, the urine or the liver extract were first passed through an OASIS cartridge. It had been demonstrated [20] that OASIS offers higher recoveries than other commercial cartridges when drugs have to be extracted from fatty tissues extracts, thus OASIS HLB (60 mg) was used for this particular purpose and the MISPE procedure described in section 2.5 was then applied.

Urine was selected as the first biological fluid for study and a series of experiments carried out aiming at the extraction of ENRO from 25 ml of this sample matrix. Figure 3a shows the chromatogram arising from a blank urine sample that has passed through the OASIS and MIP cartridges without a clean-up step being applied. When 6 ml of ACN/acetic acid (95:5) was used in a washing step, the matrix interferences were considerably reduced (Figure 3b) enabling the identification and quantification of ENRO.

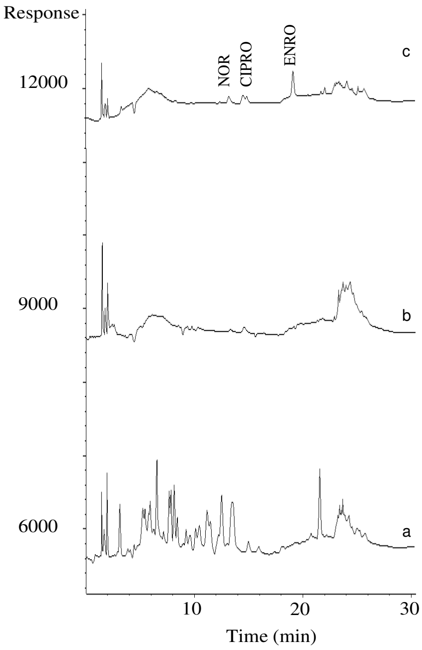


Figure 3. Chromatograms obtained from the percolation of 25 ml of urine through the OASIS cartridge and the MIP cartridge. a) blank of urine; b) blank urine when a clean-up with 6 ml ACN/acetic acid (95:5) was performed; c) urine spiked at 15 μg l^{-1} with norfloxacin, ciprofloxacin and enrofloxacin and with a clean-up step involving 6 ml of ACN/acetic acid (95:5).

In a subsequent experiment, 25 ml of urine spiked with the mixture of quinolones at 15 μg l^{-1} was pre-concentrated (Figure 3c) and ENRO was recovered at 80%. Although an impurity co-eluted with CIPRO, it was possible to quantify the quinolone and it was found that the recoveries for this analyte and NOR were lower than 35%. The ACN/acetic acid wash allowed us to not only reduce the cross-reactivity of the MIP, but also to eliminate all the matrix interferences which interfere with the quantification.

The linearity of the method was evaluated for ENRO. To establish the linear range, 25 ml of urine, which did not contain any quinolone, was spiked with ENRO at concentrations between 120 and 5 μg l^{-1}. The MISPE method was then applied and good linearity was obtained with a determination coefficient (r^2) greater than 0.9999. The repeatability for 25 ml of spiked urine (15 μg l^{-1} of ENRO), expressed as RSD (n=5), was lower than 4%.

The ability of the MIP to extract quinolones from tissue samples was also investigated. In veterinary use, ENRO must be quantified at the MRL levels, which are described by the EU Council Regulation 2377/90 [2]. In pig liver for the sum of ENRO and CIPRO the MRL level is 200 µg/kg. The two-step SPE method described previously for use with urine samples was used once more, however, in this case the percolation of the sample through the MIP was sufficient in itself to obtain clean chromatograms, making a subsequent clean-up step unnecessary (Figure 4a and b). Moreover, loses of CIPRO due to the washing solvent were also avoided.

To demonstrate the feasibility of using the two-step MISPE method to extract ENRO and CIPRO from tissue samples at lower MRL levels (200 µg/kg), 5 g of pig liver was treated using the protocol described in the Experimental section. In a first instance, the method was evaluated only to check the performance of the MIP when an extract from a tissue sample was percolated, for this reason the tissue extract and not the initial amount of tissue, was spiked with different volumes of standard solutions of NOR, CIPRO and ENRO at 100 mg l^{-1}, to obtain spiked extracts at 500, 200, 100 and 50 µg/kg. Following the two-step SPE, CIPRO and ENRO were recovered at 80% and 96%, respectively (values for spiked extract at 100 µg/kg). Moreover, it was also possible to determine NOR, the quinolone forbidden in veterinary use (recoveries about 70%).

In view of the good results obtained, the two-step MISPE method was then applied to the whole extraction procedure. Therefore, the 5 g of tissue was spiked with different volumes of standard solutions of NOR, CIPRO and ENRO. The liver was homogenised and the resultant extract passed through the OASIS and MIP cartridges (Figure 4c).

In this case, a slight decrease in the recoveries of the analytes was observed due to the tissue treatment. Thus, NOR was almost completely lost and ENRO and CIPRO were quantified at MRL/2 with a linear response for both quinolones and a determination coefficient (r^2) greater than 0.999 which demonstrates clearly that the application of MIP columns to the selective extraction of analytes from heterogeneous biological matrices is both effective and practicable.

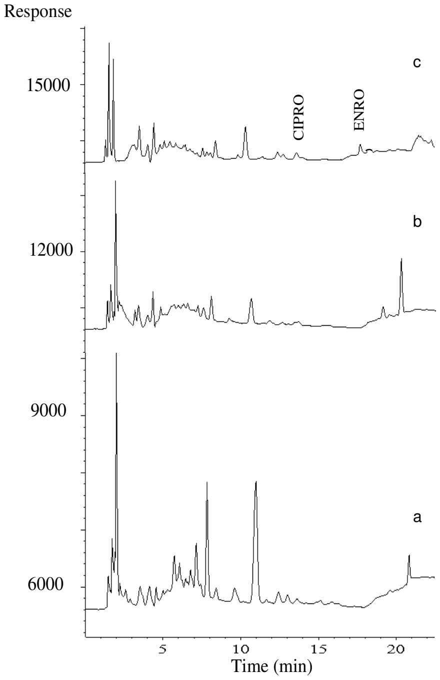


Figure 4. Chromatograms obtained from the extraction of ciprofloxacin and enrofloxacin from tissue samples. a) Blank tissue. 10 ml of tissue extract was percolated through the OASIS cartridge; b) blank tissue. 10 ml of

tissue extract was percolated through the OASIS cartridge and MIP cartridge; c) chromatogram obtained when 5 g of tissue was spiked with the mixture of quinolones at 100 µg/Kg.

CONCLUSIONS

This paper describes the first synthesis and evaluation of an enrofloxacin imprinted polymer. The chromato-graphic evaluation of the polymer established the imprinted properties of the MIP and revealed a high degree of cross-reactivity for the structurally related analyte ciprofloxacin. These attractive features were exploited in an application where the MIP was used in combination with an OASIS cartridge as SPE sorbents to clean and extract quinolones from complex biological samples. The ability of the MIP to selectively recognise quinolones from a mixture of widely used antibiotics, such as TCs, has also been demonstrated. Moreover, a two-step SPE method has been developed for the extraction of ENRO from urine and ENRO and CIPRO from tissue samples. This efficient method allowed cleaner extracts to be obtained and interfering peaks arising from the complex biological matrices to be suppressed. Of particular note is the fact that this method appears to be very well-suited indeed for the control of ENRO and CIPRO in pig liver at values lower than MRL levels.

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2.5.2 *Direct determination of ciprofloxacin by mass spectrometry after two-step solid-phase extraction using a molecularly imprinted polymer*

DIRECT DETERMINATION OF CIPROFLOXACIN BY MASS SPECTROMETRY AFTER TWO-STEP SOLID-PHASE EXTRACTION USING A MOLECULARLY IMPRINTED POLYMER

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Abstract

A molecularly imprinted polymer (MIP) has been prepared for the first time with ciprofloxacin (CIPRO) as the template molecule by a non-covalent synthetic procedure. Prior to it use a sorbent in solid phase extraction (SPE), the MIP was chromatographically evaluated which revealed a good imprinting effect. This MIP was used to selectively extract CIPRO from urine samples by a two-step SPE in which a commercial OASIS cartridge and a molecularly imprinted solid-phase extraction (MISPE) cartridge were combined. This SPE procedure allowed the matrix compounds to be effectively removed. The clean urine extracts obtained after this two-step SPE made possible to directly inject the extract to a mass spectrometer to determine the presence of CIPRO in urine samples at low levels.

Keywords: molecularly imprinted polymer; solid-phase extraction; fluorinated quinolones; human urine; mass spectrometry

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INTRODUCTION

Over the last few years, molecular imprinting technology has been widely exploited to prepare imprinted polymers, which have been mainly used as sorbents in solid-phase extraction procedures (SPE). The use of molecularly imprinted polymers (MIPs) in SPE is very useful when analysing complex samples such as biological (urine, plasma, serum, tissue) since the selectivity of these imprinted materials allows a better cleaning of the matrix sample to be achieved.

It has been demonstrated in several

studies that MIPs can be successfully applied to the selective extraction of the target analyte from complex biological samples [1-7]. In nearly all these studies [1-6], the molecularly imprinted solid-phase extraction (MISPE) has been used before a liquid chromatographic system (LC). There are only few studies in which MISPE has been used before a detection system [7-14], which is very useful because the time of analysis can be reduced. When a selective elution of the target analyte can be achieved [7-11] or the clean-up performed by the MIP is so effective that allows the interferences (which can hinder the quantification of the target analyte) to be completely removed [12-14], then the separation system can be avoided. Mullet *et al*. developed for the first time a pulsed elution (PE) [8] and differential pulsed elution (DPE) [7,9-11] systems which allowed a selective elution of the target analyte. Nevertheless the number of studies in which the chromatographic system can be avoided because a clean extract is obtained is still limited [12-14]. The first study was performed by Sellergren in 1994 [12] in which a MIP imprinted with pentamidine was directly coupled to a UV detector. In the study developed by Xie *et al*. [13] and Zhu *et al*. [14] flavonoids are isolated from herb extracts. The eluate collected after the MISPE procedure was so clean that it was possible to directly inject it in a mass spectrometer detector.

In the present paper a novel MIP material prepared with ciprofloxacin (CIPRO) as the template molecule has been used as selective sorbent in the SPE. CIPRO is a fluorinated quinolone generally used to treat urinary infections in human medicine [15]. A two-step SPE method combining an OASIS cartridge with the MIP cartridge has been established in order to selectively extract CIPRO from human urine samples. Due to the clean extracts obtained after this two-step SPE, the LC system could be avoided and the extracts collected from the extraction procedure directly injected into the MS.

EXPERIMENTAL

Reagents and standards

The chemicals used for the polymer synthesis were ciprofloxacin (CIPRO), which was kindly donated by Cenavisa (Reus, Spain), methacryclic acid (MAA) and ethylene glycol dimethacrylate (EGDMA), from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium), and dichloromethane (DCM) from Rathburn Chemicals (Walkerburn, U.K.). The monomers were purified prior to use *via* standard procedures in order to remove stabilisers, and the solvent dried over 4 Å molecular sieves. The AIBN was recrystallised from acetone.

To prepare the mobile phases or the solutions used in the SPE process we used acetonitrile (ACN) (SDS, Peypin, France), chloroform (Rathburn Chemicals), water from a Millipore water purification system (Milli-Q water), phosphoric acid (Probus, Barcelona, Spain), sodium dihydrogen phosphate (Panreac, Barcelona, Spain), acetic, formic and hydrochloric acids from Probus, sodium hydroxide (NaOH) (Prolabo, Fontenay, France) and triethylamine (TEA) (Aldrich).

Other fluorinated quinolones were used to investigate the selectivity of the MIP for structurally related compounds to CIPRO. Thus, norfloxacin (NOR) from Sigma (Steinheim, Germany), enrofloxacin (ENRO) kindly donated by Cenavisa, and the quinolone flumequin (FLU) from Sigma, were utilised. Standard solutions of each compound were prepared monthly at concentration of 100 mg l^{-1} in NaOH 0.1 M.

Another group of antibiotics such as tetracyclines (tetracycline (TC), oxytetracycline (OTC), 4-epichlorotetracycline (EPI) and doxycycline (DC)) all them from Sigma were also used in some experiments.

Preparation of the Imprinted Polymer.

A ciprofloxacin-imprinted polymer was prepared with CIPRO as the template (0.21g, 0.58 mmol) dissolved in DCM as the porogen (6.66 ml) in a 25 ml thick-walled glass tube. Then, MAA as the functional monomer (0.40g, 4.64 mmol), EGDMA as the cross-linking monomer (4.60g, 23.20 mmol) and AIBN as the initiator (0.08g, 0.51 mmol) were added and the polymerisation mixture was cooled on an ice bath and sparged with oxygen-free nitrogen for five minutes. The tubes were sealed and heated in a water bath at 58ºC for 24h. After this period, the glass tube was smashed and the monolithic polymer obtained was ground mechanically and sieved using acetone to obtain regularly sized particles with diameters between 25 and 38 µm suitable for the MISPE evaluations.

A control non-imprinted polymer (NIP) was prepared following the same procedure but with omission of CIPRO.

Instrumentation

Prior to any use in SPE, the retention capacity and selectivity of the MIP was assessed by high performance liquid chromatography (HPLC). Consequently, stainless-steel HPLC columns (15 x 0.46 cm i.d.) were slurry packed with the ground polymer (MIP or NIP) particles (25-38 µm) using an air-driven fluid pump (Haskel) with acetone as the slurrying and packing solvent at 2500 psi. An SP 8800 ternary HPLC pump with an automatic injector and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used in this pre-screening work.

For the two-step MISPE of urine samples, a commercial SPE sorbent, such as Oasis HLB (3 ml, 60 mg) (Milford, MA, USA), was first used and the eluate collected from this commercial cartridge was then applied through the MIP contained in a polyethylene SPE cartridge (6 ml, 200 mg). All the cartridges were coupled to a manifold supplied by Teknokroma (Barcelona, Spain) connected to a vacuum pump.

The liquid chromatographic system consisted of two LC-10AD pumps, a DGU-14A degasser, a CTO-10A oven and an SPD-10A UV spectrophotometric detector from Shimadzu (Tokyo, Japan). The injection volume was 20 µl and the analytical column was a 15 x 0.46 cm i.d. Eclipse XDB-C8, 5 µm, supplied by Agilent Technologies (Barcelona, Spain).

The MS analyses were performed in a flow injection analysis (FIA) mode. The instrument utilised was a chromatographic system HP1100 series LC-mass selective detector (Agilent Technologies) with an electrospray (ESI) interface and equipped with an automatic injector, a degasser and a quaternary pump.

Chromatographic Conditions

HPLC was used as a primary tool for the evaluation of the imprinting effect, for this reason, before any injection, columns were washed with a mixture of chloroform/acetic acid (95:5 *(v/v)*) to eliminate unreacted monomers arising from the synthesis until no signal was observed.

Then, columns packed with the MIP and NIP were washed with the mobile phase chloroform/acetic acid (90:10). When a stable base line was obtained, 20 µl of 10 mM CIPRO in chloroform/acetic acid (90:10) and 1 µl of the void marker (acetone) were injected. The analysis was performed in isocratic mode at 1 ml min^{-1}. The UV detector wavelength was set at 278 nm and the analysis performed at room temperature.

For the chromatographic conditions in MISPE experiments the mobile phase was a mixture of ACN (solvent A) and an aqueous phase (solvent B), which contained 0.02 M of sodium dihydrogen phosphate and 2% of TEA. The pH of the aqueous phase was adjusted to pH 3.0 with phosphoric acid and filtered through a 0.22 µm membrane filter. The flow-rate of the mobile phase was 1 ml min^{-1} and the gradient profile was from 5 to 10% B in 5 min, to 10.5% B in 11 min, to 20% in 5 min, to 50% in 2 min and then isocratic elution for a further 2 min. The column temperature was 35 ºC and all compounds were detected at 280 nm, except FLU which was detected at 324 nm.

For the MS analysis the mobile phase was ACN/acetic acid pH 3 (1:4) based on a previous work [16]. The mass spectrometer operated in positive mode simultaneously acquired in full scan (from 200m/z to 400 m/z) and under selected-ion monitoring (SIM) acquisition mode. The parameters of ESI-MS were optimised for ciprofloxacin and were the following: the drying gas was operated at a flow-rate of 13 l min^{-1} and 350°C, the nebulizer pressure was set at 40 p.s.i. and the capillary voltage was at 2000 V. The fragmentor voltage was operated at 100 V, and at 125 V for confirmation purpose. The ions acquired for ciprofloxacin were 332, 314 and 231.

MISPE Conditions

The two-step MISPE conditions for the analysis of urine samples were based on the good results obtained from a previous study developed by our research group to extract ENRO from this sample matrix [17]. Thus, 25 ml of urine (adjusted to pH 3 with HCl) was percolated through the OASIS cartridge previously conditioned with 5 ml of MeOH and 2 ml of Milli-Q water. After sample application, a washing step with 2 ml of Milli-Q water was applied and the analytes then desorbed using 10 ml of MeOH. This methanolic solution containing all the retained compounds from the commercial cartridge was then passed through the

MIP, previously conditionated with 6 ml of MeOH and then a clean-up step with an organic solvent (specified later) was performed. The retained analytes were desorbed with 3 ml of ACN/4% formic acid (1:4) and 20 µl of sample was injected onto the analytical column. For the MS analysis 20 µl of sample was injected to the ESI-MS by FIA.

Analysis of urine samples

Urine was collected from a healthy volunteer and kept in the freezer at -20ºC until their use. The urine was filtered through a 0.22 µm syringe filter before SPE process.

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

The imprinting effect of the MIP was first checked by a chromatographic evaluation. To this end, the procedure described in section 2.4 was followed. In these conditions, it was observed that the use of chloroform/acetic acid (90:10) allowed the chromatographic peak shape and retention time to be improved. When less amount of acetic acid was added, very broad chromatographic peak was observed and longer chromatographic analysis time, which confirms that hydrogen bonds were the dominant interactions in the re-binding step. From the retention times of CIPRO in the MIP and NIP the capacity factors (K'_{CIPRO}MIP= 3.95 K'_{CIPRO}NIP= 0.045) and the imprinted factor (I_f= 87.78) were calculated

To check the selectivity of the MIP for other structurally related analytes ENRO was also injected onto the MIP and NIP polymers. The capacity factors (K'_{ENRO}MIP= 1.278 K'_{ENRO}NIP= 0.031) and the imprinted factor (I_f= 40.831) were calculated in a similar manner to those for CIPRO. The Normalised Retention Index (RI) [18]. was calculated from these results. RI enables the K' values of CIPRO and ENRO to be compared RI of the template is 1 by definition and in this case the RI value for the ENRO was 0.47, which demonstrates that the MIP shows higher affinity for the template molecule.

The chromatographic evaluation verifies the selectivity of the MIP for the molecule used during the imprinting process. Moreover, the low RI value for the analogue of the template indicates that this MIP could show low cross-reactivity levels. The MIP chromatographically evaluated was then applied as sorbent in SPE in order to exploit its selectivity to the selective extraction of CIPRO from complex matrices.

MISPE

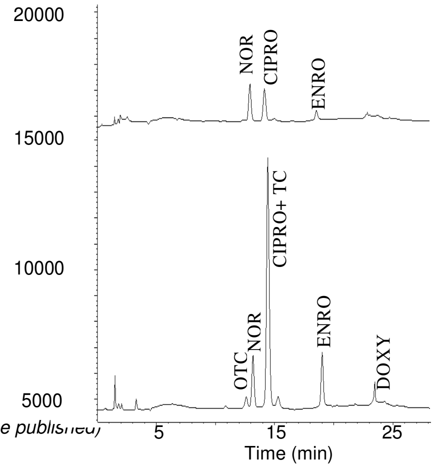
To determine the selectivity of the MIP in SPE and achieve good recoveries for the analyte of interest, the conditioning, loading and elution steps must be first optimised. In a previous study developed by group to extract ENRO from urine and tissue samples [17], it was observed that the MIP showed the best recognition properties when the sample was percolated in MeOH. The same behaviour was observed for the MIP prepared in the present study, thus, 10 ml of sample containing 0.3 mg l^{-1} of three FQs (NOR, CIPRO and ENRO) and a quinolone

(FLU) was prepared in MeOH and percolated through the MIP previously conditioned with MeOH. All the compounds were completely retained on the polymer, except FLU which was not retained during this loading step because lacks a piperizinyl ring [17]. The retained analytes were quantitatively recovered when 3 ml of ACN/4% formic acid (1:4) was used in the elution step.

From the chromatographic results it was expected that the MIP showed lower cross-reactivity levels. For this reason, in order to enhance the selectivity of the MIP a clean-up step with an organic solvent was included. Several solvents, such as DCM, ACN, toluene and ethyl acetate were investigated as a possible washing solvents resulting in maximum selectivity of the MIP. Although different volumes of these solvents were used the analytes were still retained on the MIP. Nevertheless, when 4 ml of a polar non-protic, solvent such as dimethylformamide (DMF) was applied, ENRO was only retained in a 25%, while CIPRO and NOR were still bound in a 70% and 85%, respectively. It should be noted that in these conditions, the analytes were also completely removed from the control polymer. In order to decrease the cross-reactivity of the MIP for NOR, acetic acid was added to the organic solvent; however, the selectivity of the MIP was not improved. For this reason, DMF was used for further experiments.

The selectivity of the MIP was also explored for another group of antibiotics extensively used to also treat human diseases such as TCs. 10 ml of sample spiked at 0.3 mg l^{-1} with a mixture of TC, OTC, EPI, DC and the fluorinated quinolones NOR, CIPRO and ENRO was prepared in MeOH and percolated through the MIP. TCs were only retained in about 40% because they were not able to establish the specific interactions with the MIP. Therefore, the clean-up step involving 4 ml of DMF removed all the TCs from the MIP and only the fluorinated quinolones remained still bound to the MIP as it is shown in Figure 1.

The focus of the present work was to use the MIP as SPE sorbent to clean and enrich biological samples. For this reason, the MISPE protocol was tested for the extraction of CIPRO from human urine samples.



J. Chromatogr. A (2005) (to be published)

Figure 1. Chromatograms obtained from the percolation through the MIP of 10 ml of sample prepared in MeOH containing 0.3 mg l^{-1} of a mixture of several fluorinated quinolones and tetracyclines. a) When a clean-up step was not performed; b) with a clean-up step using 4 ml of DMF.

Analysis of Biological Samples

In the previous study developed by our group [17], it was demonstrated the feasibility of the two-step SPE procedure, which allowed the biological sample to be passed through the MIP in an organic solvent, since it has been demonstrated that in this way the imprinting properties of these MIPs are enhanced. Moreover, this two-step MISPE procedure also allows matrix interferences arising from biological matrices to be effectively removed. In this study, this feature was exploited to quantify CIPRO in human urine.

For the two-step MISPE method, the procedure described in section 2.5 was then applied. Figures 2a and 2b show the chromatograms obtained after percolating a blank of urine, without and with the clean-up step with 4 ml of DMF, respectively. In a subsequent experiment, 25 ml of urine spiked with 15 µg l^{-1} of each compound was pre-concentrated (Figure 2c) and after the clean-up with DMF, CIPRO was recovered in 80%, whereas NOR and ENRO in 50% and 20%, respectively. As can be seen in Figure 2c the clean-up with DMF allowed CIPRO to be quantified because all the matrix interferences were almost completely removed. This demonstrated that the MIP was still able to exhibit the strongest affinity to its template in a complex biological system. This two-step MISPE also allowed the sample to be percolated in MeOH, so that the selective interactions between the analyte and the MIP are enhanced. Moreover, due to the extracts passed through the MIP cartridge are so clean, the MIP can be reutilised more than 100 times.

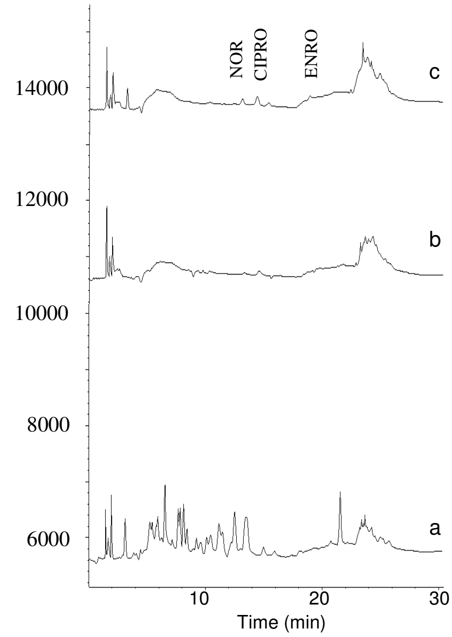


Figure 2. Chromatograms obtained from the percolation of 25 ml of urine through the OASIS cartridge and the MIP cartridge. a) blank of urine; b) blank urine when a clean-up with 4 ml DMF was performed; c) urine spiked at 15 µg/l with norfloxacin,

ciprofloxacin and enrofloxacin and with a clean-up step involving 4 ml of DMF.

The linearity of the method with UV detection was evaluated for CIPRO. To check the linear range, 25 ml of urine, which did not contain any FQ, was spiked with CIPRO at concentrations between 120 and 7 μg l^{-1}. Good linearity was obtained with a determination coefficient (r^2) greater than 0.9932. The repeatability for 25 ml of spiked (15 μg l^{-1} of ENRO) urine, expressed as RSD (n=3), was lower than 11%.

MISPE and ESI-MS

Since the extract of the samples after the OASIS-MIP extraction was quite clean, the next step was to eliminate the chromatographic separation and, therefore, the extract was directly injected by FIA to the ESI-MS operating in positive mode. The full scan spectra of ciprofloxacin, enrofloxacin and norfloxacin were different and therefore the ciprofloxacin could be quantified without interference. The ions selected for the SIM acquisition at 100 V of fragmentor voltage for ciprofloxacin were 332 (100%), 314 (15%) and 231 (10%) corresponding to the $[MH]^+$, $[MH\text{-}H_2O]^+$ and $[MH\text{-}H_2O\text{-}C_3H_4\text{-}NC_2H_5]^+$, respectively. In order to confirm the presence of the ciprofloxacin, a higher fragmentor voltage was applied, 125 V, since the fragmentation was different (332 (100%), 314 (65%), 288 (20%) and 231 (15%)). The 288 ion corresponds to $[MH\text{-}CO_2]^+$ fragment. The spectrum (from 200 to 400 m/z) for CIPRO at 100 V and 125 V are shown in Figure 3. In order to quantify from the FIA-MS analysis, different standard solutions of ciprofloxacin were analysed and the base peak 332 was quantified. Good linearity (r^2>0.990) was obtained between 1 and 500 μg l^{-1} of CIPRO.

When a blank urine sample was spiked at different concentrations of ciprofloxacin (15 μg l^{-1} and 60 μg l^{-1}) the recoveries were 80% and 87%, respectively; therefore, no significant matrix suppression was observed which is typical in MS due to the matrix compounds [19]. This may be explained because of selective extraction after the OASIS-MISPE. The relative standard deviation (%RSD n=4)) for urine samples spiked at 15 μg l^{-1} was 12.3%.

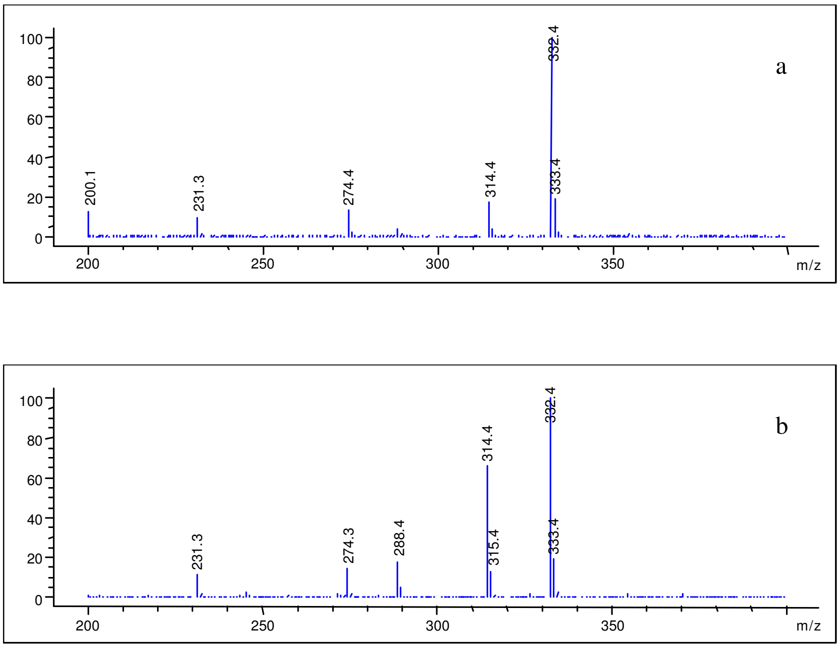


Figure3. Full scan Spectra for ciprofloxacin at different fragmentor voltage a) 100 V and b) 125 V.

CONCLUSIONS

In this study ciprofloxacin imprinted polymer has been synthesised and applied for the first time a as selective sorbent in SPE. Prior to it use, a chromatographic evaluation of the MIP allowed the imprinting effect and the cross-reactivity levels for another structurally related compound (ENRO) to be evaluated. From these results it was expected a high selectivity of the MIP for the template in SPE applications. Nevertheless it was necessary to include a clean-up step to enhance the selectivity of the MIP. When this MIP was applied in combination with an OASIS cartridge to the extraction of CIPRO from urine samples, a clean chromatogram was obtained after the optimised clean-up, which allowed the matrix compounds to be almost completely removed. Because of the efficient two-step MISPE method it was possible to directly inject into a MS the urine extracts to determine CIPRO at µg l^{-1} levels, avoiding the chromatographic column and reducing the time of analysis.

Acknowledgements

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3. *RESULTS & DISCUSSION*

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

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

As can be seen in Table 3.1 several porogens, functional monomers, T:M:X ratios and polymerisation conditions were used. It should be noted that these parameters depended on the characteristics of the template molecule used in each case (e.g. the polarity, type and number of functional groups, and the stability of the template under the UV-light). Nevertheless, the crosslinking agent and the initiator used were the same in all the MIPs prepared. EGDMA was the crosslinking agent used because it makes possible to obtain MIPs with good thermal and mechanical properties and is compatible with nearly all the organic solvents. In this Thesis it has also been demonstrated that EGDMA can be used when the porogen is a mixture of water and an organic solvent such as methanol, and the methanol/water ratio is lower or equal to 4:1 as has been previously explained for the 1-NS MIP. AIBN was used as the initiator in all the syntheses because it can react thermally and under UV light, and can therefore be used in both polymerisation methods.

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

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

^{a}template:functional monomer:crosslinker ratio $^+$Methacrylic acid *4-vinylpyridine

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

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

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

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

Table 3.2 Selectivity shown by some MIPs for several template analogues

Template	Analogue	RI $_{template}$	RI $_{analogue}$
4-CP	4-NP	1	0.93
Ibuprofen	Naproxen	1	0.90
Naproxen	Ibuprofen	1	0.75
ENRO (DCM)	Ciprofloxacin	1	4.33
ENRO ($CHCl_3$)	Ciprofloxacin	1	1.33
CIPRO	Enrofloxacin	1	0.47

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

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

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

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

This table shows that nearly all the MISPE studies were performed in off-line mode, which allows a wide variety of eluting solvents to be used. The MISPE studies were applied to the extraction of several analytes from river water and biological samples. Depending on the matrix sample, different sample pretreatments were used. For river water, the sample was just filtered through a membrane filter before being analysed. Then, the river water was directly applied through the MIP sorbents, which proved to have high affinity for the target analyte/s even in aqueous samples.

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

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

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

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

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

For the MISPE of biological samples, the clean-up step was very useful, mainly when tissue samples were analysed. To remove the matrix sample from the MIP, solvents such as ACN were used. This is a polar but non-protic solvent which removes the matrix interferences without disrupting the selective interactions between the naproxen or the OTC, and their respective MIPs. Sometimes, modifiers such as acetic acid were added to the washing solvent to decrease the cross-reactivity of the MIP, as was observed in the ENRO MIP when urine samples were analysed. However, the cross-reactivity of this MIP was very useful when fluorinated quinolones had to be extracted from tissue samples because in this way the MIP selectively extracted CIPRO and ENRO and both analytes could be quantified as specified by the European legislation. $CHCl_3$ and DMF were also used as washing solvents (see Table 3.3). After an optimization step, it was seen that they were the best washing solvents for removing the matrix sample and enhancing the selectivity of the MIP. One example is the CIPRO MIP in which clean extracts were obtained and directly injected into the mass spectrometer without signal suppression. In this way, the separation system was avoided and the time of analysis decreased.

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

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