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

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.

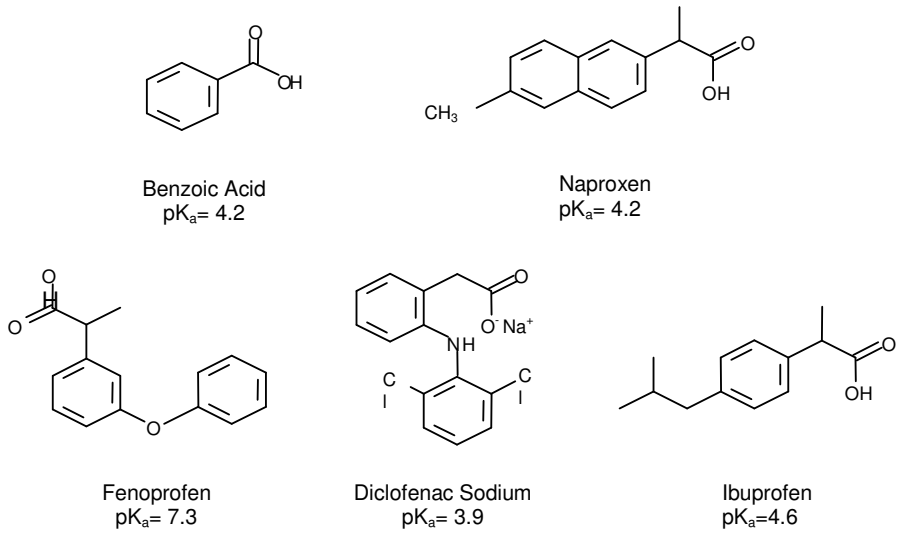


Figure 1. Chemical structures of the benzoic acid and the non-steroidal anti-inflammatory drugs used to probe the selectivity of the MIP.

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

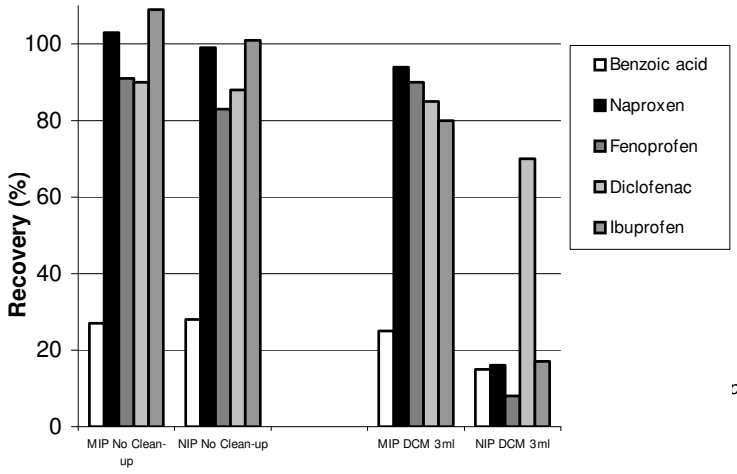
a group of polar pollutants including nitro- and chlorophenols, were added to the sample. Thus, when 10 ml of acidified Milli-Q water (pH 3) spiked with 15 µg l^{-1} of these compounds was percolated through the cartridge, all the compounds were retained on the MIP by non-specific, hydrophobic interactions. 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.



ɔress)

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)

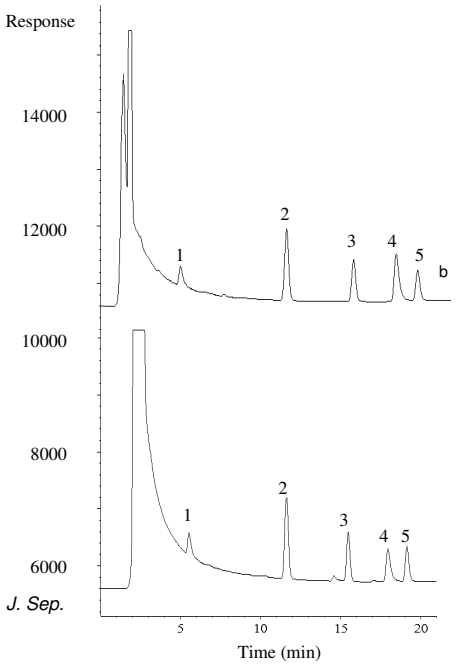


Figure 3. Chromatograms obtained by MISPE of 1000 ml of acidified Ebro river water (pH 3) spiked at 0.5 μg l^{-1} with naproxen and 2.25 μg l^{-1} with benzoic acid and the other NSAIDs. a) Without washing step; b) with a washing step using 3 ml of DCM. Peak assignation: (1) benzoic acid, (2) naproxen,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

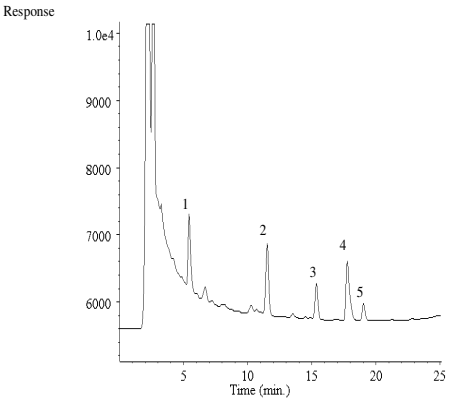


Figure 4. Chromatograms obtained by MISPE of 250 ml of acidified WWTP water (pH 3) spiked at 4 μg l^{-1} with naproxen and 18 μg l^{-1} with benzoic acid and the other NSAIDs when a clean-up step with 3 ml of DCM was performed. Peak assignation as per Figure 3.

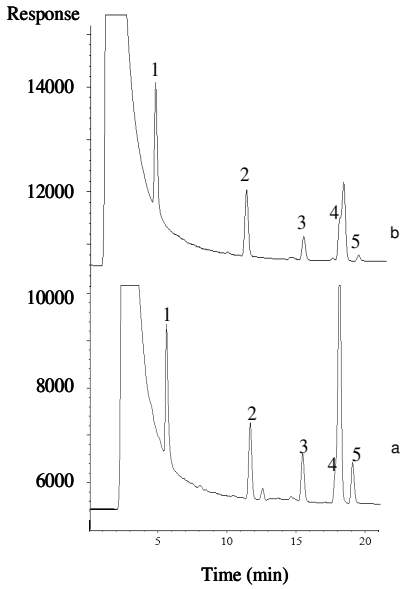


Figure 5. Chromatograms obtained after passing 1000 ml of acidified Ebro river water (pH 3) spiked at 0.5 μg l^{-1} with naproxen and 2.25 μg l^{-1} with benzoic acid and the other NSAIDs. a) through an Isolute ENV+ cartridge when a clean-up step was not performed. b) through an Isolute ENV+ cartridge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

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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.

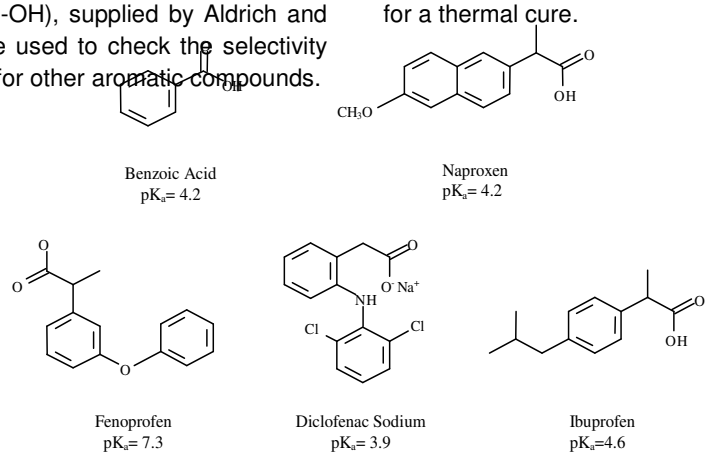


Figure 1. Chemical structures of the non-steroidal anti-inflammatory drugs used to probe the selectivity of the MIP.

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.

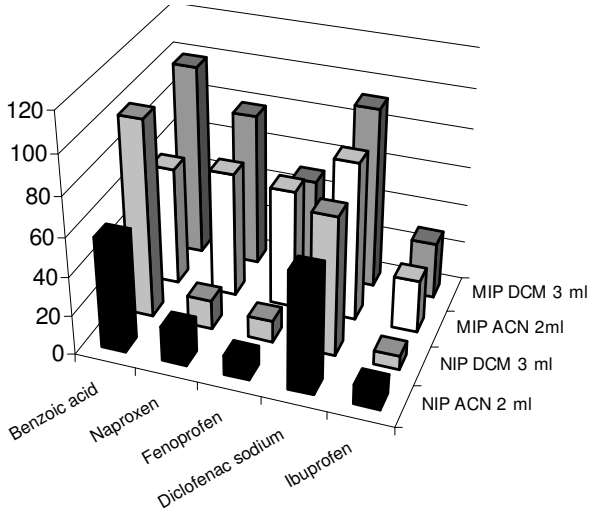


Figure 2. Selectivity of the molecularly imprinted polymer (MIP) and the non-imprinted polymer (NIP) after a washing step with 2 ml of ACN or, alternatively, with 3 ml of DCM.

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.

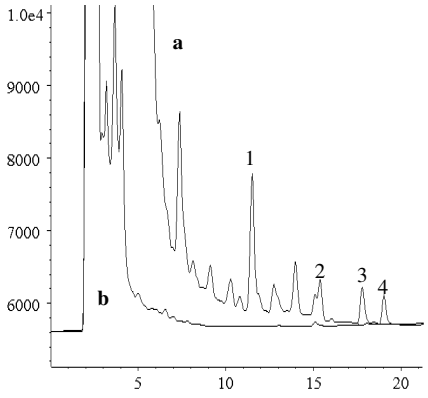
After drying the cartridge for 15 min, the effect of using DCM as washing solvent was first checked. When 3 ml of this solvent was used, the broad band at the beginning of the chromatogram was only slightly reduced; for this reason a more polar solvent (ACN) was then tested. When experiments with 2 ml 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.

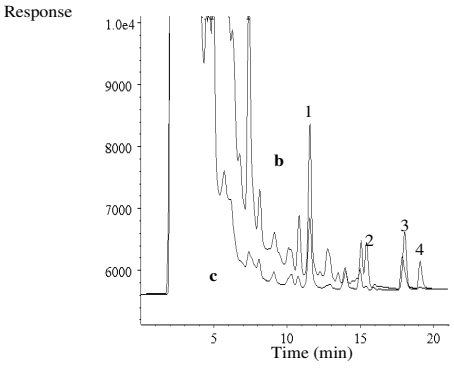
ACN was then used as the basis for further investigation.

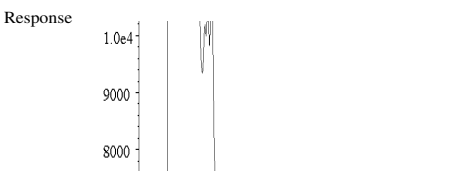
For comparative purposes experiments were also performed with a 200 mg commercial C_{18} Bakerbond (Deventer, Holland) SPE cartridge. When 25 ml of urine spiked with 55 µg l^{-1} of naproxen and 110 µg l^{-1} of the other NSAIDs was loaded, all compounds were retained on the cartridges and the recoveries 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.

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}).

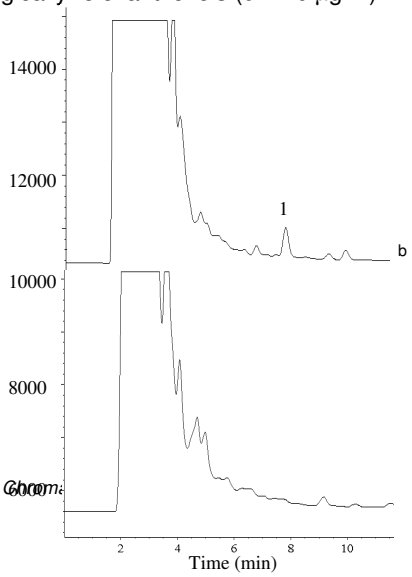


Figure 5. Chromatograms obtained upon decreasing the time of analysis. Pre-concentration of 25 ml of a urine sample (pH 3): a) without addition of naproxen, and b) spiked at 25 µg l^{-1} with naproxen and with a washing step involving 2 ml of ACN. Peak designation as per Figure 3

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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