SYNTHESIS AND EVALUATION OF A MOLECULARLY IMPRINTED POLYMER FOR SELECTIVE ON-LINE SOLID-PHASE EXTRACTION OF
4-NITROPHENOL FROM ENVIRONMENTAL WATER

ABSTRACT

A molecularly imprinted polymer (MIP) able to bind 4-nitrophenol (4-NP) was prepared using non-covalent molecular imprinting methods and evaluated as a selective sorbent in molecularly imprinted solid-phase extraction (MISPE) on-line coupled to a reverse-phase HPLC. It has been shown that the conditions chosen for washing the MIP and for eluting the analyte in the MISPE process are extremely important for ensuring good selectivity and recovery. River water samples, spiked with the eleven Environmental Protection Agency (EPA) phenolic compounds at μg l^{-1} levels, were preconcentrated on-line using this MIP and 4-NP was selectively extracted. The humic acid interference was simultaneously reduced considerably. The MIP was also compared with a commercially available highly cross-linked polymer (LiChrolut EN) and the former yielded cleaner extracts.

Solid-phase extraction (SPE) is routinely used to preconcentrate analytes of interest present at low levels of concentration, to remove interfering components from complex matrices and to change the solvent (e.g. from aqueous to organic) before chromatographic analysis. Recently, it has also been used to store and transport the analytes.

Due to the popularity of SPE, many new functionalized polymeric sorbents and highly cross-linked polymers have recently appeared as alternatives to conventional SPE materials in order to facilitate the trace enrichment of polar analytes [1,2]. Moreover, there has been a lot of recent interest in the development of selective SPE sorbents that yield cleaner extracts in the analysis of complex matrices, e.g. blood, urine and environmental water samples. One such class of selective extraction materials are the immunosorbents (ISs), which rely upon reversible and highly selective antigen-antibody interactions. They have been applied in the determination of several organic pollutants in complex environmental matrices, in both off-line and on-line methodologies [3]. Nevertheless, the difficulty and cost in obtaining biological antibodies, in addition to the other disadvantages of immunosorbents, has led to the development of synthetic antibody mimics, such as molecularly imprinted polymers (MIPs).

MIPs are cross-linked macromolecules bearing “tailor-made” binding sites for target molecules. They are prepared by the complexation, in solution, of a target compound (template) with functional monomers, through either covalent or non-covalent bonds, followed by polymerization with an excess of cross-linker to form a highly cross-linked polymer network. Upon removal of the template molecule from the polymer network, specific recognition sites that are complementary to the template in terms of their size, shape and functionality are exposed. MIPs show high selectivity in rebinding the template in preference to other closely related structures. Important considerations in the design of these polymers have been reviewed by several authors [4-6].

MIPs are being exploited in an increasing number of applications that include their use as “tailor-made” separation materials, as antibody/receptor binding site mimics in recognition and assay systems, as enzyme mimics for catalytic applications, as recognition elements in biosensors as well as in facilitated chemical synthesis [7-12]. To date, their most extensively investigated application has been as separation materials for the analysis of numerous compounds such as drugs [13,14], pesticides [15,16] and amino acids [17] in techniques such as liquid chromatography [13,15,16], thin-layer chromatography [17] and capillary electrochromatography [14]. The good selectivity obtained with MIP-based separation materials led to them being considered as promising selective sorbents for SPE. They are as a result now being extensively investigated as highly selective SPE sorbents for the washing and the preconcentration of samples prior to analysis. As mentioned above, molecularly imprinted solid phases for extraction are an alternative to immunosorbents in SPE.

Recent developments in MISPE have been reviewed by several authors [18,19]. MISPE has been used to determine drugs in complex biological fluids [20-24], nicotine in chewing gum and tobacco [25,26], bentazone in water [27] and triazine herbicides in beef liver [28], water [29,30], apple extracts and urine [30]. However, to our knowledge, all the MISPE procedures described to date have been used only in an off-line mode to a chromatographic system, except for the work of Ferrer *et al*. [1] and Bjarnason *et al*. [30]. These authors performed a first on-line SPE of triazines from complex samples with a C_{18}-silica precolumn, then all the extracted species were eluted to a MIP column by means of acetonitrile. The triazines were selectively retained on the MIP column resulting in the relief of the matrix and improvement of the subsequent chromatogram (acquired in an on-line chromatographic system with a C_{18} column). Moreover, the use of MISPE with complex environmental samples is rare and indeed is still under development.

It is known that on-line SPE procedures overcome some of the drawbacks associated with off-line SPE. For example, there is no sample manipulation between the preconcentration and the analysis steps, so loss of the analyte and the risk of contamination are reduced and the detection limits and the reproducibility improved. Furthermore, the whole sample extract enters the analytical column, so the sample volume can be smaller, the consumption of organic solvents is lower and the potential for automation is improved.

The present paper describes the synthesis and the evaluation of a non-covalently imprinted MIP as a selective SPE sorbent, on-line coupled to a liquid chromatographic system, to selectively enrich 4-nitrophenol (4-NP) from environmental water samples. 4-NP can be toxic even at low concentrations, especially to aquatic organisms. For this reason it is regulated by the Environmental Protection Agency (EPA). MIPs imprinted against any of the eleven priority EPA phenolic compounds have not been prepared before, except by Joshi *et al.* [31] who synthesized a MIP selective for phenol via a covalent approach to remove phenol impurities from anisole.

EXPERIMENTAL SECTION

Chemicals

The chemicals for the polymer syntheses were 4-nitrophenol (4-NP), 4-vinylpyridine (4-VP) and ethylene glycol dimethacrylate (EGDMA) from Aldrich (Steinheim, Germany), 2,2'-azobisisobutyronitrile (AIBN) from Acros Organics (Geel, Belgium) and acetonitrile from Rathburn Chemicals (Walkerburn, UK). The monomers were purified prior to use via standard procedures in order to

remove stabilizers. The AIBN was recrystallized from acetone and the acetonitrile dried over molecular sieves. **Safety:** special precautions are required when handling 4-VP because it is toxic and may cause burns and sensitization by inhalation and in contact with skin.

The HPLC-grade solvents were purchased from either Rathburn Chemicals or Merck (Darmstadt, Germany) and the water collected from a Millipore water purification system (Milli-Q water). The acetic and chlorhidric acids were from Probus (Badalona, Spain). The structurally related phenolic pollutants used to investigate the selectivity of the polymers were 4-chlorophenol (4-CP) and the eleven priority EPA phenolic compounds: phenol (Ph), 4-nitrophenol (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).

Apparatus

To evaluate the polymers in analytical columns, ground polymer particles were suspended in chloroform by sonication and then slurry packed into 15 x 0.46 cm i.d. stainless steel HPLC columns at 2500 psi using an air-driven fluid pump (Haskel) with acetone as the solvent. An SP 8800 ternary HPLC pump and an SP 8450 UV detector (Spectra-Physics, Mountain View, CA, USA) were used. The on-line SPE coupled to a liquid chromatograph utilized a Must column-switching device (Spark Holland, Emmen, The Netherlands), a Waters (Milford, MA, USA) M45 pump and 10 x 3 mm i.d. stainless steel precolumns, laboratory-packed with circa 40 mg of the in-house synthesized polymers. The chromatographic system 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). The injection loop volume 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).

Preparation of the Imprinted Polymer

For the preparation of the 4-NP imprinted polymer, the template (4-NP) (0.280 g, 2 mmol) was dissolved in acetonitrile (10 ml) in a 25 ml thick-walled glass tube. The functional monomer (4-VP) (0.850 g, 8 mmols), the cross-linking monomer (EGDMA) (8.000 g, 40 mmols) and the initiator (AIBN) (0.185 g, 1.12 mmols) were then added to the above solution. The solution was cooled on an ice bath, sparged with oxygen-free nitrogen for five minutes, the glass tube sealed under nitrogen and then placed in a water bath at 60 ºC. The reaction was allowed to proceed for 19 hours. A reference, non-imprinted, polymer that did not contain any template was prepared simultaneously using the same protocol. The hard polymers that were obtained were crushed, ground and wet-sieved using acetone to obtain regularly sized particles between 25 and 38 μm suitable for the chromatographic and MISPE evaluations.

Chromatographic Conditions and On-Line SPE Procedure

The chromatographic evaluation of the polymers was carried out using acetonitrile/acetic acid 99.9/0.1 *(v/v)* as the mobile phase at 0.5 ml/min. 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 acetonitrile (containing 1% (*v/v)* acetic acid) as solvent B. Both solvent A and solvent B were mixed prior to reaching the analytical column.

The flow-rate of the mobile phase was 1 ml/min 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.

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 was applied to the conditioned precolumn and the polymer then washed with 0.4 ml dichloromethane and 2 ml Milli-Q water (pH 2.5). The retained analytes were desorbed using solvent B alone and in the backflush mode to reduce band-broadening, and then transferred on-line to the analytical column [32].

RESULTS AND DISCUSSION

Chromatographic Evaluation of the Polymers

The analytical columns packed with the polymers were washed on-line with acetonitrile/acetic acid (99.7/0.3 *(v/v)*) until a stable baseline was obtained. They were then evaluated under the chromatographic conditions described earlier by injecting 10 mg l^{-1} solutions of phenol, 4-chlorophenol and 4-nitrophenol as test analytes onto the columns. The three phenolic compounds gave different retention times on the non-imprinted control column, and for this reason the data obtained in these analyses was normalized by calculating the Normalized Retention Index (*RI*). The *RI* gives a measure of the degree of recognition for a given analyte. The template molecule gives a value of 1 by definition, and other, less strongly retained, compounds give smaller values [33]. The *RI* is defined as:

$$RI = \frac{K'analyte(MIP)/K'analyte(control}{K'template(MIP)/K'template(contro}$$

where *K′* is the capacity factor.

To calculate *K′*, the dead time of the MIP and the control columns was measured by injecting acetone as a void marker. Table 1 shows the *K′* values for the test phenolic compounds in both columns and the corresponding *RI* values. 4-CP and Ph give rise to smaller *RI* values than 4-NP, and this result verifies the imprinting effect.

Table 1
Capacity factors (*K′*) and normalised retention indices (RI) obtained from HPLC evaluation of the polymers

Compound	*K′* (MIP)	*K′* (control)	*RI*
Ph	0.53	0.47	0.69
4-CP	0.89	0.71	0.77
4-NP	1.90	1.17	1.00

On-line MISPE

The polymers in the precolumns were initially washed with solvent B (acetonitrile containing 1% *(v/v)* acetic acid) to remove any residual template that could potentially interfere with the analysis, until 4-NP was no longer detectable in the eluate. The next step was to optimize the SPE process and to compare the performance of the imprinted and non-imprinted polymers in the extraction of the eleven priority EPA phenolic compounds.

The extraction step from water involved passing 10 ml of spiked (10 μg l^{-1} of each analyte) Milli-Q water, adjusted to pH 2.5 with HCl, through the precolumn. However, the preconcentration of the analytes was not selective for 4-NP without a subsequent washing process to remove the non-specifically bound compounds, because all the compounds bound to the MIP in a reverse-phase fashion, except for PCP which was not retained by the polymers. A suitable (normal-phase) washing process would remove the non-specifically bound compounds from the MIP, whilst the 4-NP would remain bound. In this way the 4-NP could be selectively extracted. So, in the first instance, three organic solvents, acetonitrile, ethyl acetate and dichloromethane, were tested as washing solvents to remove the non-specifically bound analytes.

In spite of the fact that neither ethyl acetate nor dichloromethane is miscible with water, the Must column-switching device used in the MISPE allowed use of both solvents without any problem [32]. When the precolumn was washed with 0.2 ml acetonitrile, the recoveries of all the analytes decreased considerably. With 0.4 ml acetonitrile the 4-NP neither could be determined selectively because various analytes gave small signals, and with higher volumes of acetonitrile all the phenolics were washed off from the precolumn, including the 4-NP. However, when 0.1 ml ethyl acetate was passed through the precolumn, all phenolics were washed off, except for 4-NP, 2,4-DNP and 2-M-4,6-DNP. These three bound phenols were then eluted onto the analytical column, and the recoveries were found to be 20, 52 and 36%, respectively. With higher volumes of ethyl acetate all the phenols were washed off the polymer. Nevertheless, when the same volume (0.1 ml) of dichloromethane was used to wash the polymer, and the bound analytes subsequently eluted, only one intense signal was observed (4-NP), whilst the other phenolic analytes gave rise to small signals or indeed no signal at all, showing that a selective washing protocol had been discovered. Dichloromethane was therefore selected as the washing solvent of choice, and

the next step was to find a suitable volume of dichloromethane to selectively extract 4-NP.

Different volumes (0.2, 0.4 and 0.6 ml) of dichloromethane were investigated in the selective washing step. The results are shown in Table 2. 4-NP was not selectively retained on the MIP with 0.2 ml dichloromethane because three compounds, 4-C-3-MP, 2,4-DCP and 2,4,6-TCP, also appeared in the subsequent elution chromatogram, although the other analytes had been successfully removed. However, when the precolumn was washed with 0.4 or 0.6 ml of dichloromethane only one peak appeared in the subsequent elution chromatogram, and this corresponded to 4-NP. These results are shown in Figure 1.

Table 2
Recoveries (%) obtained by washing the 4-NP-imprinted polymer with different volumes of dichloromethane following the preconcentration of 10 ml of a standard solution spiked at 10 μg l^{-1} with all analytes [a]

Analyte	Volume CH_2Cl_2 (ml)				
	0	0.1	0.2	0.4	0.6
4-NP	76	73	67	58	50
4-C-3-MP	59	48	33	--	--
2,4-DCP	48	28	15	--	--
2,4,6-TCP	38	12	3	--	--

[a] %RSDs were lower than 7 in all instances *(n=4)*

The non-imprinted polymer was used to extract the same sample in the same manner, but when 0.4 ml of dichloromethane was applied as a washing solvent, no peaks appeared in the elution chromatogram. These results clearly

demonstrate the desired difference in selectivity between the imprinted and the non-imprinted control polymer. Figure 2 illustrates the distinctly behavior of the non-imprinted polymer. From an analytical point of view, 0.4 ml was selected as the ideal volume of dichloromethane for selective washing of the MIP, because with higher volumes the recovery decreased (see Table 2). The dichloromethane washing step eliminates the non-specific interactions between the analytes and the polymer. This fact explains why the recovery of 4-NP is decreased when a washing step is employed. So, for a selective MISPE, lower recoveries are obtained.

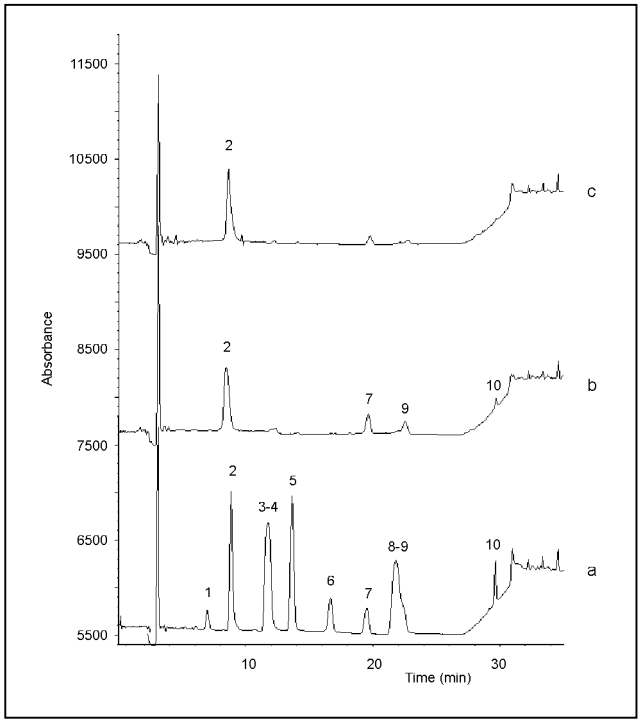

Fig. 1. Chromatograms obtained by on-line SPE with the 4-NP-imprinted polymer of 10 ml standard solution (pH 2.5) spiked at 10 μg l^{-1} with each phenolic compound. (a) without washing step and (b,c) with washing step using 0.2 and 0.4

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.

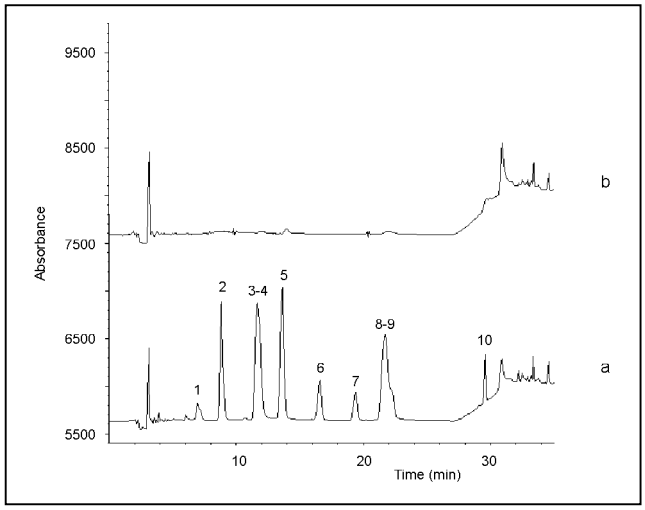

Fig. 2. Chromatograms obtained by on-line SPE with the non-imprinted polymer of 10 ml standard solution (pH 2.5) spiked at 10 μg l^{-1} with each phenolic compound. (a) without washing step and (b) with washing step using 0.4 ml of dichloromethane. Peak designation: as per Fig. 1.

Dichloromethane has low polarity and aprotic properties, so the MIP operation mode changes from reverse-phase mode during the aqueous sample loading to affinity mode in the selective washing with dichloromethane. In the affinity mode hydrogen bonding could be the dominant interaction responsible for specific retention of 4-NP on the MIP.

The effect of the sample pH on the extraction process was also evaluated. The sample pH was adjusted to 9 in order to exploit potential ionic interactions between the phenolate forms of the analytes and the nitrogen atoms in the pyridine residues of the polymer, in an attempt to increase the recovery of 4-NP.

However, the extraction with the imprinted polymer was not selective, even after a washing step with 0.4 ml of dichloromethane. The analytes that could be detected were 4-NP, 2,4-DNP, 2-M-4,6-DNP, 2,4,6-TCP and PCP, with recoveries 4, 66, 75, 26 and 42%, respectively. These results suggest that at pH 9 non-specific ionic interactions are more important than any specific interactions arising from imprinting. As a result, the subsequent analyses were all performed at pH 2.5.

To confirm that the acetic acid in the elution solvent (solvent B) had an important role to play in desorbing 4-NP from the MIP, pure acetonitrile was tested as the elution solvent. Under these conditions the 4-NP recovery decreased from 58% to 39% (%RSD was 4 for 4 analyses). Thus, the addition of acetic acid results in an increase in the recovery of 4-NP. The most likely explanation is that acetic acid competes with 4-NP for the functional groups in the binding sites, and in doing so desorbs the 4-NP from the MIP.

The breakthrough volume for 4-NP in the MIP was determined under the optimum conditions. This was carried out by percolating through the MIP different volumes (10, 15 and 25 ml) of standard solutions spiked with different concentrations of analytes, such that the amount of each analyte in the samples injected was constant (0.1 µg). The 4-NP recoveries were 58, 44 and 38% (%RSDs were lower than 8 for 4 analyses) for the 10, 15 and 25 ml sample volumes respectively. From these results, it can be concluded that 10 ml is a suitable sample volume because with higher sample volumes the recovery decreased. To obtain a higher recovery, less dichloromethane had to be used, but under these conditions other analytes were also recovered (see Table 2). Further studies to increase the capacity of this imprint, and thus the sensitivity of the method, are in progress.

MISPE of Real Water Samples

The potential value of the imprinted polymer for on-line MISPE of 4-NP in complex matrices was investigated. The complex matrix chosen was Ebro river water (TOC content 2.5-3.5 mg l^{-1}) which commonly contains humic acids. Humic acids can prevent detection of polar compounds because a broad band due to the presence of these acids elutes early in chromatograms [2]. To address this matrix interference problem, we tested the MIP with Ebro river water samples and compared its performance with a commercially available highly cross-linked polystyrene-divinylbenzene resin, LiChrolut EN (Merck, Darmstadt, Germany). The difference in performance between the 4-NP MIP and LiChrolut EN in the analysis of 10 ml of Ebro river water spiked at 10 µg l^{-1} with each of the phenolic compounds is shown in Figure 3. SPE was carried out under the optimum conditions for both sorbents. As can be seen in Figure 3b, none of the analytes could be determined using LiChrolut EN when the washing step was employed. However, with the MIP the 4-NP was selectively extracted (Figure 3a). This result also confirms the existence of specific interactions between 4-NP and the MIP arising from imprinting. The washing step with dichloromethane enabled the humic acids band to be reduced but, obviously, LiChrolut EN could not be washed with dichloromethane because the 4-NP was eluted simultaneously. It should also be noted that the MIP gave a slightly narrower interfering matrix signal than LiChrolut EN when the washing process was not carried out (see Figures 3c and d). In spite of the higher selectivity of the MIP, the recovery of 4-NP was only 36% (with the washing step) (Figure 3a) in contrast to 75% for LiChrolut EN (without the washing step) (Figure 3d) (%RSDs were lower than 7 for 4 analyses). Thus, more development work is needed to improve this methodology, and in particular to improve capacity so that lower detection limits can be reached.

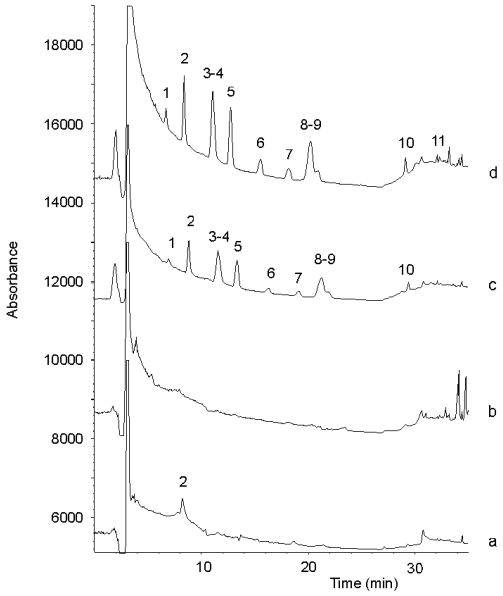

Fig. 3. Chromatograms obtained by on-line SPE with the 4-NP-imprinted polymer (a,c) and LiChrolut EN (b,d) of 10 ml Ebro river water (pH 2.5) spiked at 10 μg l^{-1} with each phenolic compound. (a, b) with washing step using 0.4 ml of dichloromethane and (c,d) without washing step. Peak designation: (11) PCP, others as per Fig. 1.

Finally, it is worth noting that the MIP precolumn was used to preconcentrate at least 70 water samples with no noticeable deterioration in performance, which augurs well for the future of MISPE.

CONCLUSIONS

The results obtained during the course of this research clearly demonstrate the value of the non-covalent imprinting technique for producing “tailor-made” sorbents for SPE, and in particular for the environmental pollutant 4-NP. This research also demonstrates the practicality of on-line coupling MISPE to a liquid chromatographic system, where the analyte is selectively extracted using only a MIP, an approach that has not hitherto been demonstrated. The application of on-line MISPE to environmental water samples confirmed the ability of the MIP precolumn to selectively isolate 4-NP from other matrix components. This result addresses an important characteristic disadvantage of the commercial sorbents commonly used for this analyte, i.e. non-selectivity. In contrast to the MIP, the non-imprinted control polymer and LiChrolut EN did not show specificity towards 4-NP, although careful selection of the SPE parameters was necessary to achieve selective extraction with the imprinted polymer. Further optimization of the MIP synthesis may improve the capacity of the MISPE column and, consequently, allow lower 4-NP concentrations to be determined.

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