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Synthesis and characterisation of molecularly imprinted polymers for the selective extraction of oxazepam from complex environmental and biological samples

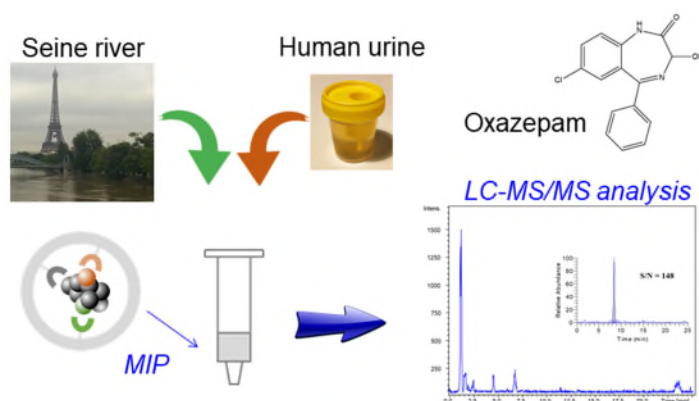
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Abstract

Oxazepam, one of the most frequently prescribed anxiolytic drugs, is not completely removed from waste waters with conventional treatment processes. It can thus also be found in environmental waters at trace levels, human urine constituting the major source of contamination. This study focussed then on the development and characterisation of molecularly imprinted polymers (MIPs) for the selective solid-phase extraction of oxazepam at the trace levels from environmental water and human urine samples. Two MIPs were thus synthesized and their selectivity in pure organic and aqueous medium were assayed. After optimizing the extraction procedure adapted to a large sample volume to reach a high enrichment factor, the most promising MIP was applied to the selective extraction of oxazepam from environmental waters. Extraction recoveries of 83 ± 12 , 92 ± 4 and $89 \pm 10\%$ were obtained using the MIP for tap, mineral and river water respectively while a recovery close to 40% was obtained on the corresponding non-imprinted polymer (NIP). Thanks to the high enrichment factors obtained, the LOQ was of 4.5 ng L^{-1} for river water. A selective extraction procedure was also developed for urine sample and gave rise to extraction recoveries close to 95% on MIP and of only 23% on NIP, which. Using the MIP, a LOQ of 357 ng L^{-1} for oxazepam in urine was obtained. The use of the MIP also allowed to limit matrix effects encountered for the quantification of the oxazepam in environmental samples and in human urine samples after extraction on Oasis HLB sorbent.

Key words

Benzodiazepines, Molecularly imprinted polymers, Solid phase extraction, LC-MS/MS, Environmental water samples, Human urine samples.

1. Introduction

Oxazepam (OXA) is one of the most marketed among active benzodiazepines (BZDs) available in France for its anxiolytic properties [1] (Seresta[®], 374 million of marketed tablets in 2015 [2]), and is also one of the active metabolites of other BZDs [3]. It is detected in urine at concentration levels ranging from a few $\mu\text{g L}^{-1}$ to a few mg L^{-1} in urine [4–9]. Human biological fluids as urine constitute the major source of contamination of OXA in environmental waters. It is a halogenated compound with a core structure consisting in the association of a benzene and a diazepine rings (**Fig. 1**), which made it resistant to the photodegradation [10] and then persistent in influents and effluents of treatment plants and surface waters. Moreover, most of sewage treatment plants are not efficient for the total removal of OXA, which explain why 80% of OXA may remain in treated wastewaters [11]. OXA has been found in influents, treated effluents from treatment plants and surface water at concentrations ranging from a few ng L^{-1} to a few $\mu\text{g L}^{-1}$ [12–16] in Europe. This could therefore constitute a risk for aquatic animals, to develop a pharmacological response to OXA, and ultimately for human health [17].

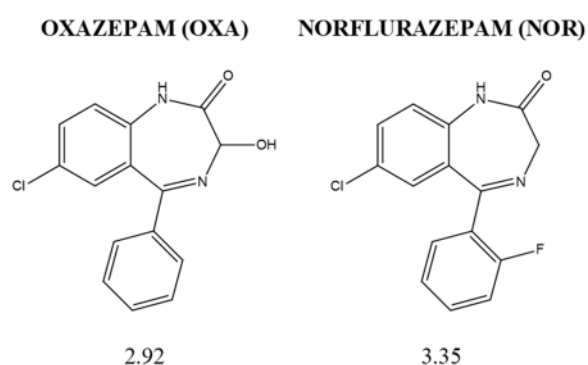


Fig. 1. Chemical structure and octanol-water partition coefficient (log P) of OXA and NOR.

As other BZDs, the analysis of OXA in environmental waters [12, 13, 16, 18] is mainly achieved by liquid chromatography coupled to mass spectrometry (LC-MS). Due to the low concentration levels in environmental waters and biological fluids, a sample treatment step by solid-phase extraction (SPE) [12, 13, 18–21] must be included in the analytical procedure in order to concentrate it. However, SPE performed with conventional sorbents (C18 silicas, polymers) can lead to the co-extraction of compounds with similar physico-chemical properties and thus to co-elution in liquid chromatography which may affect the reliability of the method. An alternative to these sorbents is to use more selective materials such as molecular imprint polymers (MIPs) which have cavities that allow selective trapping of a given compound or compounds of close structure. Basically, the synthesis of a MIP is performed in the presence of a template molecule that is removed after synthesis, thus releasing cavities that are complementary in size, shape and chemical function to the template molecule. The nature and the strength of the interactions between targeted compounds and MIPs depend on the nature of the monomer, of the cross linker and of the porogen used during the synthesis process. To assess the selectivity of the retention on MIP, a non-imprinted polymer (NIP) can also be synthesized under the same conditions but without the introduction of the template molecule.

Some studies focused on the selective extraction of BZDs from biological samples including hair [22, 23], serum [24, 25], plasma [26–28] or urine [25, 28] using MIPs introduced directly as particles or fibres into samples for partitioned extraction [24, 26, 28] or as particles introduced into cartridges for exhaustive extraction (SPE) [22, 23, 25, 27]. Of these studies, only two involved the selective extraction of OXA, the main subject of these studies being the selective extraction of diazepam (DIA) and its metabolites from hair extracts [22, 23]. These works highlighted the potential of MIPs developed for DIA to extract OXA with a high extraction recovery (73–74 %) [22, 23]. However, the evaluation of the potential of these MIPs was performed for hair extracts reconstituted in a small volume of toluene.

The potential of a MIP capable of selectively extracting OXA from environmental waters has not yet been demonstrated. For this, considering the low levels of concentration (ng L^{-1}) it is important to be able to percolate larger volumes of samples in order to concentrate the targeted compounds and thus to

facilitate their further quantification in LC-MS/MS. Until now, only one study focused on the selective extraction of DIA and lorazepam from environmental waters [21]. It was carried out using a commercialized MIP whose conditions of synthesis were only partially described, only the nature of the used monomer being mentioned (methacrylic acid, MAA). Moreover, this study did not concern OXA. All these MIPs used the targeted BZDs as template molecule such as DIA [22, 23, 25–27] or clonazepam [24, 28]. However, it has been shown that the analysis of drugs at trace levels in environmental water samples or biological samples requires the selection of a dummy template to avoid false positives and/or overestimation of drug quantities due to the risk of template leakage when applying the MIP to real samples [29, 30]. The use of a structural analogue as template was only described for the development of a MIP-based sensors to determine OXA in pharmaceutical formulation [31].

The present work aimed to evaluate the potential of MIP to selectively extract OXA which is one of the most marketed BZDs in France and an active metabolite of other BZDs. In order to avoid the risk of leak of the template when applying the MIP to real biological or environmental samples, a BZD which is not prescribed in France and not included in the list of prioritized pharmaceutical compounds that should be monitored in French environmental waters [32] was used as dummy template: norflurazepam (NOR).

Two MIPs were then synthesized by using MAA, *i.e.* the most used monomers for the synthesis of MIP for BZPs, and by varying the nature of the cross-linking agent. The selection of the best synthesis conditions was achieved by comparing the retention of OXA in pure organic solvents and aqueous media on each MIP and on their corresponding NIPs. After optimization of the extraction procedure, the MIP giving rise to the best selectivity for OXA was studied in more detail by evaluating its ability to extract it from large volumes of several water samples such as drinking, mineral, and river water. The potential of this MIP to extract OXA from another complex aqueous sample, *i.e.* urine, was then investigated. Finally, the performance of the MIP in terms of extraction yield and clean-up efficiency for a river water sample and a urine sample was finally compared to that of the commercial sorbent, Oasis HLB polymer. During these different parts of the study, the behavior of OXA-MIP, towards others BZDs (listed as priority pharmaceutical compounds to be monitored in French environmental waters) like bromazepam (BRO), alprazolam (ALPRA), diazepam (DIA) and lormetazepam (LORM) was also studied.

2. Materials and methods

2.1. Materials

NOR (96%) was provided by ABCR (Karlsruhe, Germany). ALPRA (analytical standard) was from Carbon Scientific (London, UK). DIA (> 98.5%), LORM (> 98.5%) and OXA (> 98.5%) were supplied by Lipomed (Weil am Rhein, Germany). MAA (99%), EGDMA (98%), divinylbenzene (DVB, 80%), acetone (ACS reagent, > 99.5%), hexane (anhydrous, 95%), ammonium hydroxyde solution (33%), azo-N,N'-bis(isobutyronitrile) (AIBN, 98%) and BRO (analytical standard) were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). MAA and EGDMA were distilled under vacuum to remove inhibitors. Acetonitrile (ACN, HPLC-grade-ACS reagent), dichloromethane (DCM, HPLC-isocratic-stabilized with ethanol), methanol (MeOH, HPLC-grade), toluene (HPLC-grade), heptane (HPLC-isocratic grade) and formic acid (FA, for analysis-ACS reagent, 99%) were provided by Carbo Erba (Val de Reuil, France). Hydrochloric acid (for analysis-ACS reagent, 32%) and ammonium acetate (crystal extra pure) was from Merck (Darmstadt, Germany). Acetic acid (AA, glacial, anhydrous for analysis, 100%) was from VWR (Fontenay-sous-Bois, France). Ultrapure water was provided by a Milli-Q purification system from Millipore (Saint Quentin en Yvelines, France). A mixture of the six BZDs at the same concentration (5000 µg L⁻¹) in ACN was used as stock solution to prepare the standard solutions and the solutions of percolation for SPE. This mixture and the standard solutions were stored at -20°C until use and the solutions of percolation were prepared extemporaneously.

2.2. Instrumentation and analytical conditions

For small percolated volume (1 mL), the analyses of the fractions of percolation, washing and elution resulting from the use of the MIPs and the corresponding NIPs were carried out in LC-UV with a system composed of a liquid chromatograph (Agilent 1200 series system, Agilent Technology, Massy, France) equipped with a diode array detector controlled by the Chemstation software. OXA and other BZDs were separated with a C18 Omnispher column (150 x 2 mm, 5 µm, Varian, Middelburg, The

Netherlands). The temperature of the column was fixed at 35°C and the injection volume was set at 5 μL . The mobile phase consisted in a mixture of ACN/H₂O/FA (35/65/0.1, v/v/v) with a flow rate set at 0.2 mL min⁻¹. The BZDs were detected by UV absorbance at 240 nm.

For higher percolated volume (4, 15, 25 and 35 mL), LC-MS/MS analyses of the fractions of percolation, washing and elution resulting from the use of the MIPs and the corresponding NIPs were performed on the same column with a liquid chromatograph (Ultimate 3000®, Thermo Scientific, Illkirch, France) hyphenated with a triple quadrupole (TQ) mass spectrometer equipped with a heated electrospray ionization source (TSQ Quantum Access MAX, Thermo Scientific). The system was controlled with the Chromeleon, Thermo TSQ Tune Master and X-Calibur softwares. The LC conditions were identical to those applied to analyse BZDs in LC-UV. The quantification of BZDs in MS was performed with MRM detection in positive mode. The spray voltage was set at 3000 V and the vaporiser and capillary temperatures at 350°C and 200°C respectively. The pressure of the sheath gas was fixed at 40 psi. The auxiliary and the ion sweep gas pressures were set at 5 and 0 psi respectively. The value of the skimmer offset was fixed at 20 V. Nitrogen was used as desolvation gas and argon as the collision gas at a pressure set at 1.5 mTorr. Two transitions were monitored for each BZD and collision energy related to each transition was optimized, the calibration curves obtained by LC-UV and LC-MS/MS systems are given in Electronic Supplementary Material (**Tables S.1** and **S.2** respectively). The ratio between the area of the peak corresponding to the MRM transition of the quantifier and the area of the peak corresponding to the MRM transition of the qualifier ion was calculated after LC-MS/MS analysis of standard solution of the targeted compounds (Table S.2). The elution fractions resulting from the use of the MIP and of a commercial sorbent (OASIS HLB, Waters, Saint Quentin en Yvelines, France) were first analysed in MRM mode on TQ mass spectrometer in order to calculate the extraction yield for each BZD on each support. Then, the fractions were analysed with a liquid chromatograph (Agilent 1260 Infinity, Agilent Technology, Massy, France) coupled with a quadrupole-time of flight (Q-TOF) mass spectrometer equipped with an electrospray ionization source (micrOTOF-Q Brucker Daltonics, Champs sur Marne, France) in positive full scan mode ($m/z = 100 - 2000$) in order to visualize the complexity of the sample. The system was controlled with the HyStar and OtofControl softwares. The chromatographic conditions were the same as those used to analyse BZDs with LC-UV system.

2.3. Synthesis of molecularly imprinted polymers

Two MIPs, MIP 1 and MIP 2, were synthesized by bulk polymerization with a template/monomer/cross-linker molar ratio fixed at 1/4/20. MAA was used as monomer for both MIPs. The cross-linker used to prepare MIPs was EGDMA for MIP/NIP 1 and DVB for MIP/NIP 2. As DVB was envisaged, appropriate solvent consisting in a mixture of DCM and toluene was considered as porogen for both MIPs to promote polar interactions between the monomer and the template molecule and to allow the complete solubilisation of DVB [33]. Briefly, 333 μmol of NOR, 1.3 mmol of MAA, 6.6 mmol of the selected cross-linker and 80 μmol of AIBN were dissolved in a mixture consisting in 1.8 mL of DCM/toluene (46/54, v/v) in a glass tube. After vortexing, the solution was purged with a N₂ stream for 20 min and the glass tube was sealed and incubated at 56°C for 24 h. In parallel, non-imprinted polymers (NIP 1 and NIP 2) were synthesized in the same conditions but without introducing the template molecule in the polymerization mixture. Then, each polymer was crushed, ground automatically with a mixer (MIL MM 301, Retsch, Eragny sur Oise, France) and sieved using a vibratory sieve shaker (Retsch). Sedimentation of particles with size between 25 and 36 μm was performed three times with 20 mL of a mixture of MeOH/H₂O (80/20, v/v) to remove the thinnest particles. 55 mg (0.11 cm³) of dried particles of MIP or NIP were packed between two polyethylene frits (Sigma-Aldrich) in 3-mL disposable polypropylene cartridges (Sigma-Aldrich). The packed polymers were washed with 3 mL of MeOH followed with 70 mL of a mixture composed of MeOH/AA (90/10, v/v) to remove the template. The washing fractions were evaporated under N₂ stream and resuspended in 200 μL of mobile phase prior to analysis by LC-UV. The washing step was pursued until NOR was no longer detected in the washing fractions. The polymers were finally washed with 10 mL of MeOH to remove traces of AA and stored dry at 4°C until use. The synthesis of the couple MIP/NIP 1 was performed in triplicate by two different operators to investigate the reproducibility of its synthesis.

2.4. Evaluation of MIPs in pure organic media

Two extraction procedures were applied on each couple MIP/NIP to investigate the presence of cavities on MIP, an organic solvent selected to promote the same type of interaction developed during the imprinting process was used as percolation solution. The first procedure consisted in conditioning the sorbents with 3 mL of hexane. Then, 1 mL of hexane spiked at $1000 \mu\text{g L}^{-1}$ with the selected BZDs was percolated through the polymers. They were washed with 1 mL of a mixture of toluene/hexane (50/50, v/v) and the BZDs were eluted with 1 mL of a mixture of MeOH/AA (95/5, v/v). Concerning the second procedure, the cartridges were conditioned with 3 mL of toluene and 1 mL of toluene spiked at $1000 \mu\text{g L}^{-1}$ with the selected BZDs was then percolated through the sorbents. After washing the cartridges with 1 mL of DCM, the BZDs were eluted with 1 mL of a mixture of MeOH/AA (90/10, v/v).

For both extraction procedures, the elution fractions were evaporated under N_2 stream and resuspended in 500 μL of mobile phase prior to LC-UV analysis at 240 nm.

2.5. Evaluation of MIPs in pure aqueous media

The MIP and NIP cartridges were conditioned with 2.5 mL of MeOH and 2.5 mL of ultrapure water. After percolating 1 mL of ultrapure water spiked at $1000 \mu\text{g L}^{-1}$, the polymers were washed with 300 μL of HCl 0.1 M followed with 300 μL of ultrapure water. Then, the cartridges were dried under vacuum for 20 min. The polymers were washed with 1.5 mL of a mixture of DCM/hexane (70/30, v/v). The BZDs were eluted with 1.5 mL of MeOH. The elution fractions were evaporated under N_2 stream and resuspended in 200 μL of mobile phase prior to LC-UV analysis.

The repeatability of the synthesis of the MIP 1 was investigated by performing three extractions procedures on each synthesized couple MIP/NIP 1. The spiking level was set at $250 \mu\text{g L}^{-1}$. The BZDs were eluted with 1.5 mL of MeOH. The elution fractions were evaporated under N_2 stream and resuspended in 200 μL of mobile phase prior to analysis by LC-UV.

2.6. Application to environmental water samples

2.6.1. Optimization of the extraction procedure applied to large sample volume

The breakthrough volumes for both MIP/NIPs were evaluated using the same procedure described in Section 2.5 except that the percolated volume of tap water ($\text{pH} = 7.7 \pm 0.1$, conductivity = $510 \pm 1 \mu\text{S cm}^{-1}$) was varying from 15 to 35 mL and spiked with the same quantity of BZD (1 μg), the concentration level thus ranging from 67 to $28 \mu\text{g L}^{-1}$.

In order to optimize the washing conditions, the same extraction procedure described in Section 2.5 was used except that 35 mL of tap water spiked at $3.57 \mu\text{g L}^{-1}$ with each BZD were percolated on MIP 1 and the polymers were washed with decreasing volume, 1.5, 1.25 or 1 mL, of a mixture of DCM/hexane (70/30, v/v). The elution fractions were evaporated under N_2 stream and resuspended in 500 μL of mobile phase prior to analysis by LC-MS in MRM mode using the TQ analyzer.

2.6.2. Application of MIPs to mineral and river water

Seine river water was collected in Paris and filtered twice with glass-fiber filters (type 5, 0.7 μm , 4.7 cm, Sigma-Aldrich). The optimized extraction procedure was applied in triplicate on MIP/NIP 1 to extract BZDs from 35 mL of Evian water ($\text{pH} = 7.9 \pm 0.1$, conductivity = $461 \pm 1 \mu\text{S cm}^{-1}$) spiked at $3.57 \mu\text{g L}^{-1}$ and from 35 mL of Seine river water ($\text{pH} = 8.0 \pm 0.1$, conductivity = $516 \pm 1 \mu\text{S cm}^{-1}$ after filtration) spiked at 0.01, 0.5 and 1 $\mu\text{g L}^{-1}$. The sorbents were washed with 1.25 mL of a mixture of DCM/hexane (70/30, v/v). The conditioning and the elution steps were similar to those of the procedure described in Section 2.5. The elution fractions were evaporated under N_2 stream and resuspended in 500 μL of mobile phase prior to analysis by LC-MS in MRM mode (TQ analyzer) for the quantification and only for the elution fraction corresponding to percolation of Seine river water in scan mode on a Q-TOF mass analyser for the evaluation of the complexity of the sample.

2.6.3. Comparison of performances of the MIP with a conventional sorbent

The extraction procedure on OASIS HLB cartridge (3 mL, 60 mg, Waters, Guyancourt, France) was adapted from procedure described by Hummel *et al.* [34]. Briefly, the sorbent was conditioned with 600 μL of heptane followed by 600 μL of acetone, 1.8 mL of MeOH and 2.4 mL of ultrapure water. Then, 35 mL of Seine river water spiked at $0.01 \mu\text{g L}^{-1}$ were percolated through the sorbent. The cartridge was dried under vacuum for 1 h. The BZDs were eluted with 1.2 mL of acetone. The elution

fractions of OASIS HLB was evaporated under N₂ stream and resuspended in 500 µL of mobile phase before LC-MS analysis in MRM mode on TQ mass analyzer for the quantification and in scan mode on a Q-TOF mass analyser for the evaluation of the complexity of the sample.

2.7. Application of MIPs to biological fluids

2.7.1. Application of MIPs to human urine samples

The sample of urine was filtered through a 0.2 µm filter (cellulose acetate, VWR) and diluted with one volume of ammonium acetate-acetic acid buffer (0.2 M, pH 6.0). The diluted sample of urine was spiked at a concentration of 0.5, 1.5 and 15 µg L⁻¹ (equivalent in urine) with only the BZDs retained selectively (OXA, BRO and ALPRA) and vortexed for 30 s. The extraction procedure was applied in triplicate on MIP/NIP 1. The extraction procedure on the MIP and on the NIP was similar to the one described in the Section 2.6.2 except that 4 mL of the spiked diluted sample of urine were percolated through the sorbents. The elution fractions were reconstituted in 500 µL of mobile phase after evaporation under N₂ stream before analysis by LC-MS in MRM mode using the TQ analyzer.

2.7.2. Comparison of performances of the MIP with a conventional sorbent

The extraction procedure on Oasis HLB was adapted from the extraction procedure proposed by Wang *et al.* [35]. Briefly, the cartridge was conditioned with 2 mL of MeOH followed by 2 mL of ultrapure water and 2 mL of ammonium acetate-acetic acid buffer (0.2 M, pH 6.0). The filtered sample urine was diluted with one volume of ammonium acetate-acetic acid buffer (0.2 M, pH 6.0) and spiked at 0.5 µg L⁻¹ (equivalent in urine) with the BZDs retained selectively on MIP (OXA, BRO and ALPRA). Then, the mixture was vortexed for 30 s and 4 mL were percolated through the sorbent. The sorbent was washed with 2 mL of 5% MeOH (with 2% ammonium hydroxyde) followed by 2 mL of ultrapure water. The BZDs were eluted twice with 1 mL of MeOH (fractions named E1 and E2). After evaporation under N₂ stream, the elution fraction was resuspended in 500 µL of mobile phase. The elution fractions were analysed with LC-MS in MRM mode on a TQ mass analyzer.

2.7.3. Matrix effects during LC/MS-MS analysis

A blank filtered urine sample was diluted two times with ammonium acetate-acetic acid buffer (0.2 M, pH 6.0) and vortexed for 30 s. 4 mL of this solution were percolated through each sorbent. The resulting elution fractions were evaporated under N₂ stream and resuspended in 500 µL of standard of 2 µg L⁻¹ diluted in mobile phase. The extraction procedure was repeated three times. All fractions were analysed with LC-MS in MRM mode on a TQ mass spectrometer and the ratio between the area of the peak corresponding to the MRM transition of the quantifier ion and the area of the peak corresponding to the MRM transition of the qualifier ion was used to confirm the identification of each targeted compound. The matrix effects were evaluated by comparing the area of peaks of BZDs of a standard at 2 µg L⁻¹ prepared in pure media with those of the elution fractions issued of the MIP 1 or of the conventional sorbent spiked at the same concentration just before the LC/MS-MS injection. The matrix effects (ME) were defined as $ME = 100 \times \frac{\text{Area of BZD in the elution fraction}}{\text{Area of standard}}$.

3. Results and discussions

3.1. Choice of the synthesis conditions

The aim of this work was to develop a selective extraction procedure based on a MIP for the determination of OXA that should be monitored at the trace levels in environmental waters (concentrations ranging from a few ng L⁻¹ to a few hundred ng L⁻¹ in French rivers [16,32]).

The synthesis of a MIP implies the careful selection of a template molecule to lead to the formation of cavities capable of recognizing target analytes. However, when using a MIP developed for the trace analysis of a drug in environmental waters, leakage of even a small amount of the template molecule (< 4.7.10⁻⁵ % of quantity of template molecule introduced for synthesis) could lead to a false positive response. [33]. In order to circumvent this problem, a structural analogue, NOR, which is not one of the

targeted BZDs detected in French environmental water samples [32], was then selected as template molecule for the synthesis of the MIPs (see structure of NOR and OXA in **Fig. 1**). Except in one publication that used 2-(trifluoromethyl)acrylic acid as monomer [24], the MAA was used as acidic monomer in association with ethylene glycol dimethacrylate (EGDMA) as cross-linker [22, 23, 25–27] with a template/monomer/cross-linker molar ratio of 1/4/20 [24–26] or 1/6/30 [23, 27] in toluene [25] or in chloroform [22, 23, 26, 27] as porogen. Therefore, to limit the number of synthesis conditions to be screened, MAA was also selected as monomer for its ability to develop polar interactions with NOR and OXA but the effect of the nature of the cross-linker on the retention properties was investigated by synthesizing MIPs either with EGDMA (MIP/NIP 1), often associated with MAA for MIP synthesis, either with divinylbenzene (DVB), selected for its ability to develop π - π interaction with compounds having aromatic groups (MIP/NIP 2). In order to allow the complete solubilisation of the polymerization mixture, the syntheses were performed in a DCM/toluene mixture (46/54, v/v) as porogen. These non-protic solvents should promote the expected H bonds between the template and the monomer during the polymerization process.

3.2. Characterization of the MIPs in pure organic media

The potential of retention of both MIPs was first studied by percolating 1 mL of OXA and NOR *i.e.* the template molecule, diluted in hexane, a non-protic solvent selected to favour H bonds. The contribution of cavities to their retention was evaluated by the comparison of extraction yields obtained on the MIPs and their corresponding NIPs. The polymers were washed with 1 mL of a mixture toluene/hexane and both BZDs were eluted with a mixture MeOH/AA in order to disrupt expected polar interactions. The extraction yields of OXA and NOR in the elution fraction were similar (close to 100%) for MIP and NIP 1 and for MIP and NIP 2 illustrating the non-contribution of the cavities supposed to be formed in the retention process (data not shown). To limit the retention on the NIP while keeping the retention on the MIP that must be stronger than on NIP if cavities were formed, the polarity of the solvents involved in the extraction procedure has been slightly increased. Thus, hexane was replaced by toluene as percolating solvent and the washing step was performed with 1 mL of DCM, the elution step being again carried out with a mixture of MeOH/AA. Extraction yields in the percolation, washing and elution fractions are shown in **Fig. 2** for OXA and NOR but also for other BZDs that can be found in French water, *i.e.* BRO, ALPRA LORM and DIA which structure are given in supplementary material (Fig. S.1). Higher extraction yields were obtained for OXA, NOR and BRO in the elution fraction of the both MIPs than in that of the corresponding NIPs, thus indicating the presence of specific cavities in the MIPs. In contrast, ALPRA, LORM and DIA are weakly retained by both MIPs. The extraction profiles obtained for both couples of MIPs/NIPs being similar, it was not possible to select the most suitable MIP for the selective extraction of OXA at this stage of the study. As the final purpose of the study was to use the MIP synthesized to extract OXA from environmental waters, a deeper evaluation of MIPs was achieved in aqueous media.

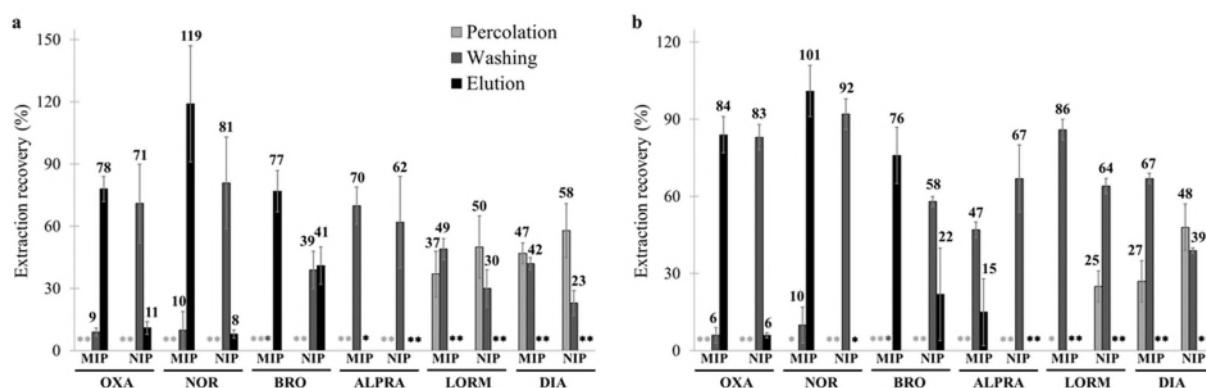


Fig. 2. Extraction profiles obtained after the percolation of 1 mL of toluene spiked at 1000 $\mu\text{g L}^{-1}$ with each BDZ on (a) MIP/NIP 1 ($n = 3$) and on (b) MIP/NIP 2 ($n = 3$). Extraction procedure: washing with 1 mL of DCM, elution with 1 mL of MeOH/AA (90/10, v/v). The quantification was performed by LC-UV at 240 nm. *: Unquantifiable ($\text{LOD} < \text{value} < \text{LOQ}$), **: Not detectable ($\text{value} < \text{LOD}$).

3.3. Retention of target analytes in pure aqueous media

It was already demonstrated by our group that, with methacrylate-based MIPs, divalent cations contained in real aqueous samples can replace the protons of the carboxylic acid groups present in MIPs during the percolation step [36]. This cation exchange can compromise the establishment of polar interactions (such as hydrogen bonds) necessary for selective retention of the target analytes on MIPs. To circumvent this problem, after the percolation of aqueous samples, the interaction sites can be regenerated by a washing of the MIPs with aqueous hydrochloric acid 0.1 M followed by ultrapure water [36]. A washing step with a non-water miscible solvent (mixture of DCM/hexane) being also envisaged, the polymers were thus dried before this washing step. This procedure was applied after the percolation of 1 mL of ultrapure water spiked at 1 mg L⁻¹ with OXA and other BZDs and the recoveries obtained for the elution fraction of MIPs and NIPs are presented in **Fig. 3**. Again, OXA and NOR are selectively retained on both MIPs with extraction yields of 85 and 95% on MIP 1 (versus only 41, and 29% for NIP 1) and 86 and 80% on the MIP 2 (versus only 57, and 28% for NIP 2) respectively. Concerning other BZDs, MIP 1 strongly retains BRO but without any selectivity, similar recoveries being obtained on MIP 1 and NIP 1. In return, the MIP 2 allows selective retention of BRO with extraction yields significantly higher on the MIP 2 (74 %) than on the NIP 2 (30 %). Conversely, a selective retention of ALPRA is only obtained on MIP 1, with extraction yields of 30 % for MIP 1 and 11% for NIP 1, similar extraction yields being obtained on MIP 2 and NIP 2 (32 and 37% respectively) for this compound. At last, the two MIPs do not allow the retention of LORM and DIA in aqueous media because they were poorly recovered in the elution fraction. In conclusion, the two MIPs do not present the same behaviour towards two studied BZDs, BRO and ALPRA. However, with regards to their behaviour towards the target analyte, OXA, it was not possible to select one. Therefore, some experiments on large sample volume of water were made.

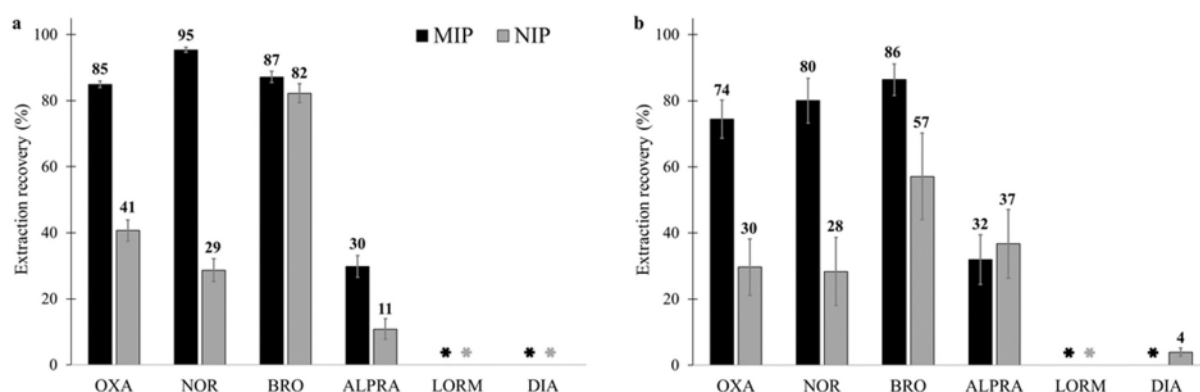


Fig. 3. Extraction recoveries in the elution fraction obtained after the percolation of 1 mL of ultrapure water spiked at 1000 $\mu\text{g L}^{-1}$ with each BZD on (a) MIP/NIP 1 ($n = 3$) and on (b) MIP/NIP 2 ($n = 3$). Extraction procedure: washing with 300 μL of HCl 0.1 M followed by 300 μL of ultrapure water, drying under vacuum for 20 min, washing with 1.5 mL of DCM/hexane (70/30, v/v) and elution with 1.5 mL of MeOH. The quantification was performed by LC-UV at 240 nm. *: Unquantifiable (LOD < value < LOQ).

3.4. Application to environmental water samples

3.4.1. Determination of the breakthrough volume

One of the objectives of this work being to selectively extract OXA from environmental samples with a high enrichment factor to improve the sensitivity of the method. Percolation of larger sample volumes may result in the early elution of OXA because of the low but non-negligible elution strength of water itself. The breakthrough volume should thus be determined by percolating increasing volume of water samples. For these experiments, spiked tap water was selected to mimic possible matrix effects (mainly caused by large amounts of divalent cations) that may occur during the analysis of real environmental waters. Increasing volumes of tap water from 1 to 35 mL spiked with 1 μg of OXA and of template

NOR were then percolated through each MIP. As the aim of these experiments was to verify the retention of both compounds during the percolation step, the elution step was performed directly after percolation step, without any washing step. The analysis of this elution fraction make it possible to estimate the losses exclusively generated by the percolation step.

Significant losses have occurred during percolation step for both BZDs on MIP 2 when increasing the percolation volume from 1 to 15 mL (from 26 to 48% of loss for OXA and 20 to 43% for NOR respectively). In return, the percolation of up to 35 mL of spiked tap water on MIP 1 does not affect recoveries in the elution fraction of the OXA and NOR. MIP 1 was then selected for the continuation of the study; it was named MIP (and NIP for NIP 1) and the percolation volume was set at 35 mL thus providing a theoretical maximal enrichment factor of 70. Moreover, before applying this MIP to various real samples, the reproducibility of its synthesis was studied.

3.4.2. Repeatability of synthesis of the MIP

The repeatability of the synthesis of the MIP was investigated by synthesizing independently two others couples of MIP/NIP. In order to reduce the percolation time and to compare the results to previous extraction profiles, the same extraction procedure as the one described in **Fig. 3** was applied in triplicate on each synthesized MIP/ by percolating only 1 mL of ultrapure water spiked at $250 \mu\text{g L}^{-1}$ with OXA and NOR. The average recoveries obtained for the elution fractions of MIP and NIP are reported in **Fig. 4**. Again, the three synthesized MIPs retained selectively OXA and NOR as the recoveries obtained with MIPs (89 and 97% respectively) were higher than those obtained with the NIPs (41 and 21% respectively). The standard deviations associated to these recoveries on the three synthesized MIPs were lower than 10% for both compounds. They were similar to those obtained for three independent extraction procedures performed on a single MIP (one-way analysis of variance: selected $\alpha = 0.01$, p-values $> \alpha$). The repeatability of the synthesis of the MIP was thus acceptable.

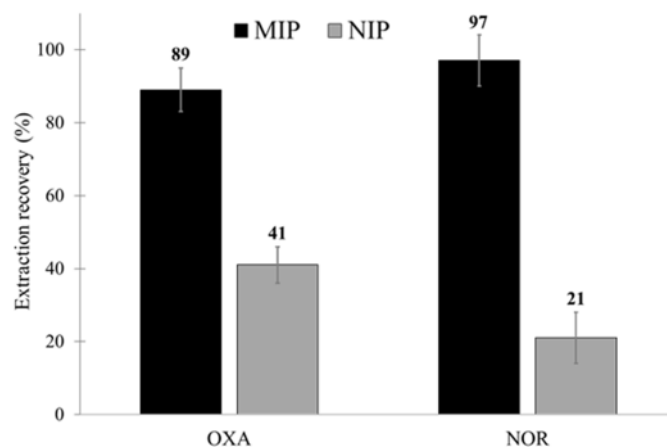


Fig. 4. Extraction recoveries in the elution fraction obtained after the percolation of 1 mL ultrapure water spiked at $250 \mu\text{g L}^{-1}$ with BZDs on three synthesized polymers MIP/NIP 1 (% \pm SD values, $n = 3$ for each synthesized sorbent). The extraction procedure was the same to the one described in Fig. 3.

3.4.3. Retention of target analytes in environmental samples

If the ability of this MIP to extract OXA from 35 mL water sample with high recoveries was demonstrated, the percolation of large volume could lead to an early elution of BZDs on the sorbents during the percolation step. It could thus be necessary to decrease the elution strength of the washing solution by reducing the volume or by modifying the composition of the washing solution in order to maintain the retention and the selectivity previously obtained for lower volumes of water (section 3.3). Therefore, the first washing steps with HCl solution and pure water were maintained as it should ensure the presence of proton on the carboxy moieties, but the various volumes of the washing solution (1,5, 1.25 and 1 mL) of the DCM/hexane (70/30, v/v) mixture was applied. which were previously selectively retained by the MIP, BRO and ALPRA. The extraction yields obtained after the percolation of 35 mL

of spiked tap water are given in **Table 1**. Imprinting factors (IFs) corresponding the ratio between recoveries on MIP and on NIP are also provided. The recoveries for OXA, NOR and BRO on MIP remain constant whatever the volume used for the washing step while the recovery of ALPRA increases while decreasing of the volume of washing solution. The volume of 1 mL has failed to provide selective retention of OXA and BRO on MIP as the IF values are close to 1. On the other hand, a washing volume of 1.5 mL is too large, as it resulted in a strong decrease of the extraction yields in the elution fraction of MIP despite the very satisfactory IF values obtained in these conditions. Thus, the volume of washing solution was set at 1.25 mL for the further studies as it led to the best compromise between selectivity and extraction yields for OXA but also for NOR, BRO and ALPRA. It is interesting to note that the modification of the extraction procedure (higher sample volume and reduction of the washing volume) has led to the selective retention of the BRO on the MIP, whereas until now the BRO has been retained in a non-selective way. The SD values of the recoveries of BZDs were less than 20% ($n = 3$) (**Table 1**) reflecting satisfactory repeatability to extract BZDs from tap water. This optimized extraction procedure was also applied to the percolation of 35 mL of non-spiked tap water sample. 3 ng of NOR were detected in elution fraction of MIP thus pointing out the residual leakage of the template used for the imprinted process. This amount corresponds to less than 4.10^{-6} % of the quantity of NOR introduced for the MIP synthesis. This clearly demonstrates the value of using an analogue as a template to avoid any risk of a false positive.

Table 1. Extraction recoveries (%) in the elution fraction and corresponding IFs obtained after the percolation of 35 mL of tap water spiked at $3.57 \mu\text{g L}^{-1}$ with BZDs on MIP/NIP and corresponding imprinting factor (IF) when applying different washing volume of DCM/hexane (70/30, v/v): 1 ($n = 1$), 1.25 ($n = 3$) and 1.5 mL ($n = 1$). The extraction procedure was the same than in Fig. 3 except for the washing step. The quantification was performed by LC-MS in MRM mode.

BZD	$V_{\text{washing}} = 1 \text{ mL}$			$V_{\text{washing}} = 1.25 \text{ mL}$			$V_{\text{washing}} = 1.5 \text{ mL}$		
	MIP	NIP	IF	MIP	NIP	IF	MIP	NIP	IF
OXA	71	61	1.2	83 ± 12	41 ± 2	2.0 ± 0.3	78	30	2.6
NOR	82	39	2.1	104 ± 14	26 ± 1	4.0 ± 0.3	115	19	6.1
BRO	98	73	1.3	88 ± 18	62 ± 6	1.4 ± 0.3	79	50	1.6
ALPRA	60	38	1.6	43 ± 6	24 ± 2	1.7 ± 0.2	28	19	1.5

This procedure was then applied to a non-spiked Evian water. Thanks to its high and constant ion content over time, this mineral water is usually used by French water suppliers as reference water to control their analytical methods. Except the leak of the template molecule (NOR) from the MIP (3 ng) observed in the elution fraction, no targeted compounds were detected in non-spiked Evian water. 35 mL of Evian water spiked at $3.57 \mu\text{g L}^{-1}$ were thus percolated through the MIP/NIP. The extraction recoveries in the elution fraction obtained for MIP/NIP are reported in **Fig. 5**. The selectivity of MIP was maintained for the extraction of OXA, NOR, BRO and ALPRA from Evian water as recoveries obtained with MIP were higher than those obtained with NIP. The SD values associated to the extraction yields of OXA and others BZDs were lower than 7% demonstrating the repeatability of the extraction procedure applied to Evian water. Altogether these results confirmed the previous results obtained for tap water in terms of extraction efficiency and selectivity.

The optimized extraction procedure was also applied to a Seine river water sample. First, a non-spiked sample of Seine river water analysed with the optimized procedure and 2.1 ± 0.3 ng of OXA were detected in the elution fraction of MIP indicating that Seine river water was contaminated by OXA¹. This amount of OXA was considered to calculate the extraction recovery resulting from the analysis of spiked Seine river water. Moreover, the template molecule NOR was excluded from this experiment achieved at very low concentration level ($0.01 \mu\text{g L}^{-1}$) as its release by the MIP (3 ng) during elution step is 8.5 times higher than the lowest percolated quantities envisaged (0.35 ng).

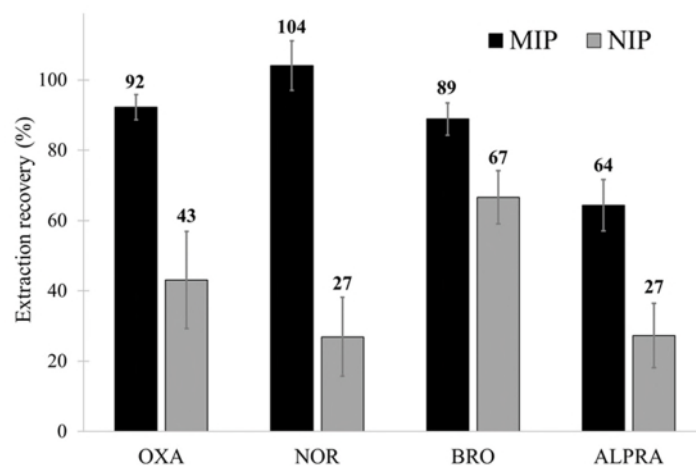


Fig. 5. Extraction recoveries in the elution fraction obtained after the percolation of 35 mL of Evian water spiked at $3.57 \mu\text{g L}^{-1}$ with each BZD ($n = 3$). The extraction procedure was the same than in Fig. 3 except that: 1.25 mL of DCM/hexane (70/30, v/v) was used for the washing step. The quantification was performed by LC-MS in MRM mode.

The optimized extraction procedure was then applied to a Seine river water sample spiked at concentration levels closed to the lowest levels of contamination of BZDs found in environmental samples including 0.01, 0.5 and $1 \mu\text{g L}^{-1}$ [12, 13, 16, 18] (**Table 2**). The results were similar to those obtained for spiked tap water (**Table 1**) or Evian water (**Fig. 5**). The selectivity was thus maintained for the extraction of OXA, BRO and ALPRA from Seine river water spiked at very low levels of concentration. The extraction recoveries for these BZDs were repeatable (standard deviations $< 20\%$ for OXA for the studied range of concentration) and similar whatever the considered spiking levels showing the linearity of the method applied to environmental water samples (one-way analysis of variance: selected $\alpha = 0.01$, p-values to OXA, BRO and ALPRA $> \alpha$). It is also interesting to note that OXA was present in the Seine river water at a concentration level of $66.2 \pm 8.8 \text{ ng L}^{-1}$.

Table 2. Extraction recoveries in the elution fraction obtained after the percolation of 35 mL of spiked Seine river water and of 2 mL spiked urine diluted with 2ml ammonium acetate-acetic acid buffer (0.2 M, pH 6.0) on MIP and NIP (% \pm SD values, $n = 3$). The quantification was performed by LC-MS in MRM mode. Concentration levels indicated for urine correspond to concentration value in urine before dilution.

Spiking level ($\mu\text{g L}^{-1}$)	BZD	MIP	NIP
River water			
0.01	OXA	89 ± 10	37 ± 12
	BRO	111 ± 32	54 ± 12
	ALPRA	61 ± 19	13 ± 3
0.5	OXA	92 ± 7	20 ± 5
	BRO	98 ± 3	43 ± 7
	ALPRA	62 ± 16	18 ± 4
1	OXA	88 ± 20	37 ± 6
	BRO	90 ± 15	60 ± 8
	ALPRA	50 ± 11	15 ± 3
Human urine			
0.5	OXA	106 ± 6	$< \text{LOQ}$
	BRO	126 ± 8	57 ± 10
	ALPRA	38 ± 11	10 ± 2
1.5	OXA	97 ± 18	26 ± 5
	BRO	103 ± 14	42 ± 3
	ALPRA	41 ± 9	16 ± 1
15	OXA	81 ± 12	19 ± 2
	BRO	93 ± 9	39 ± 5
	ALPRA	42 ± 5	18 ± 2

3.4.4. Comparison of performances with a conventional sorbent

To complete the evaluation of the potential of the MIP for the analysis of trace of OXA and other BZDs in river waters, its performance in terms of extraction yield and clean-up efficiency were compared to that provided by Oasis HLB sorbent which is commonly used to extract drugs, including BZDs, from environmental waters [13, 34]. A previously developed extraction procedure on this commercial sorbent was adapted to the percolation volume of 35 mL [35]. The recovery yields in the elution fraction obtained after the percolation of 35 mL of Seine river water spiked at $0.01 \mu\text{g L}^{-1}$ with OXA, BRO and ALPRA on Oasis HLB were 98 ± 16 , 98 ± 11 and 91 ± 13 %. This demonstrates the good performance of this widely used sorbent in terms of retention. However, the elution fraction resulting from the use of Oasis HLB sorbent was more complex than the one obtained for the MIP as indicated by the presence of many peaks on the base peak LC-MS-chromatogram in scan mode ($m/z = 300 - 2000$) (**Fig. 6 a**). In addition, the S/N ratio obtained for the LC-MS/MS analyses in MRM mode for the quantification of OXA in the elution fraction were higher ($S/N = 148$) using the MIP (**Fig. 6 b**) than using Oasis HLB sorbent ($S/N = 116$) (**Fig. 6 c**). The LOQ were 4.5, 1.6 and 1.2 ng L^{-1} for OXA, BRO and ALPRA respectively using the MIP. Thus, the MIP permitted to selectively extract BZDs from spiked environmental water samples at concentrations of only a few ng L^{-1} . Hence, treatment of the sample on MIP with LC-MS/MS analysis allows OXA to be quantified at a concentration adapted to the analysis of real environmental water samples and with a 30% increase in sensitivity as compared to the commercial Oasis HLB sorbent ($\text{LOQ} = 5.8 \text{ ng L}^{-1}$ for OXA). In addition, as illustrated by **Table S.3**, the developed analytical method requires to percolate only 35 mL of river water through the MIP to provide similar performances to others analytical methods with a percolated volume divided by a factor of 15 to 30 (percolation volumes from 100 to 500 mL).

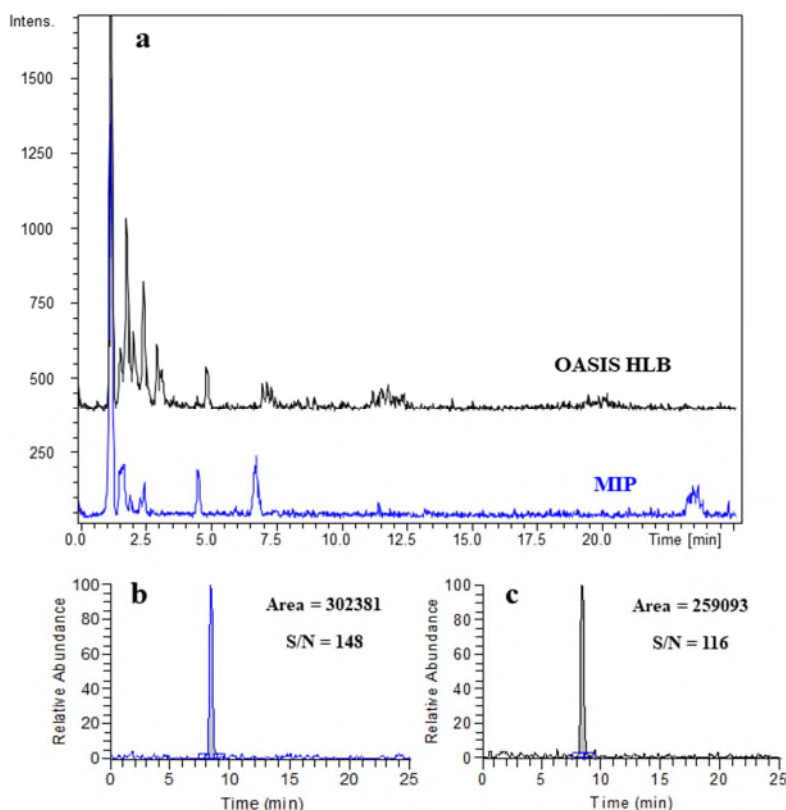


Fig. 6. Base peak LC-MS-chromatogram corresponding to the analysis of the elution fraction obtained after the percolation of 35 mL of Seine river water spiked at $0.01 \mu\text{g L}^{-1}$ with OXA, BRO and ALPRA on MIP and on Oasis HLB (a). LC-MS analysis in scan mode ($m/z = 300 - 2000$) on a Q-ToF mass spectrometer. The baseline of the chromatogram of the elution fraction obtained for Oasis HLB was artificially shifted to facilitate its reading (intensity offset: 400 a.u.). The inserts correspond to the chromatograms of the same sample provided by LC-MS in MRM mode on a TQ mass spectrometer for the quantification of OXA ($287.1 \rightarrow 241.1$) in the elution fraction of MIP (b) and Oasis HLB sorbent (c).

3.5. Application to biological samples

3.5.1. Retention of target analytes in human urine samples

Since human urine is one of the major pathways of environmental water contamination, it is necessary to be able to quantify OXA and other BZDs in this biological fluid beforehand. In urine, BZDs and metabolites are detected at higher concentration level than in environmental water, ranging from a few $\mu\text{g L}^{-1}$ to a few thousand $\mu\text{g L}^{-1}$ [4–9, 37, 38]. Thus, the followed experiments were achieved at concentration levels ranging from 0.5 to 15 $\mu\text{g L}^{-1}$ and to a reduced volume of urine sample (2 mL). The extraction yields obtained after the percolation of urine diluted with one volume of ammonium acetate-acetic acid solution (0.2 M, pH 6.0) on MIP and NIP are presented in **Table 2** for three spiking level, *i.e.* 0.5 $\mu\text{g L}^{-1}$, 1.5 and finally 15 $\mu\text{g L}^{-1}$ (equivalent in urine). It is noteworthy that the template molecule NOR was not included in these experiments, Indeed, as in the case of the analysis of Seine river water, the quantity of NOR released by the MIP during the elution step is almost 3 times higher than the lowest quantity studied (1 ng). The optimized extraction procedure was also applied to a non-spiked urine sample. No targeted compounds (OXA, BRO and ALPRA) were detected in this urine sample.

The retention and the selectivity were thus maintained for the extraction OXA and the two other BZDs from urine sample. Moreover, the extraction recoveries for these BZDs were similar whatever the considered spiking levels showing the linearity of the extraction procedure applied to human urine (one-way analysis of variance: selected $\alpha = 0.01$, p-values closed to OXA, BRO and ALPRA $> \alpha$). Extraction yields are repeatable as the SD were lower than 20% for the three BZDs. The LOQ values of OXA, BRO and ALPRA measured for human urine samples were 357, 227 and 76 ng L^{-1} respectively and below the lower concentrations of BZDs and metabolites determined in human urine (a few $\mu\text{g L}^{-1}$) and to the LOQ of already published analytical methods (**Table S.3**).

3.5.2. Comparison of performances with a conventional sorbent and evaluation of matrix effects

As for river Seine water, the performances of the MIP were compared to that of Oasis HLB[®] sorbent by percolating the same human urine sample spiked at 0.5 $\mu\text{g L}^{-1}$. Extraction yields of 123 ± 9 , 162 ± 21 and 155 ± 10 % were obtained for OXA, BRO and ALPRA respectively. These extraction yields were higher than those obtained on the MIP and well above 100%. Matrix effects affecting the quantification by LC-MS of these BZDs after the treatment of urine samples on MIP and on Oasis HLB were determined according to the procedure described in section 2.7.3 and are reported in **Table 3**. The matrix effects for OXA when OASIS HLB was used were negligible (exhausting of the signal of less than 10%). However, the use of OASIS HLB sorbent clearly led to an overestimation of the amount of BRO and ALPRA in urine samples (exhausting of the signal of 47 and 44% for BRO and ALPRA respectively) and thus to an overestimation of the extraction recoveries. Conversely, using the MIP, the matrix effects were negligible for all the BZDs (below 10%) showing that the MIP is an efficient tool to remove the interfering compounds of urine samples. It allows to overcome the problem of quantification observed when analysing the urine sample with conventional sorbents such as Oasis HLB.

Table 3. Matrix effects for BDZs for MIP and Oasis HLB sorbent (% \pm SD values, n = 3). The extraction procedure was the same than in Fig. 5. The quantification was performed by LC-MS in MRM mode.

BZD	MIP	Oasis HLB
OXA	103 ± 3	90 ± 7
BRO	109 ± 10	147 ± 10
ALPRA	105 ± 5	144 ± 19

4. Conclusion

In this work, we synthesize molecular imprinted polymers in order to selectively extract OXA from environmental waters and human urine. After the screening of some synthesis conditions and the optimization of the extraction procedure adapted to different type of samples, OXA as two other BZDs, *i.e.* BRO and ALPRA, were selectively extract with high recoveries from real water samples (mineral,

tap and river seine) and from urine samples. The interest of the use of a structural analogue of the target molecules as template molecule during the MIP synthesis was demonstrated since a slight progressive leakage of this molecule was observed when applying the MIP to the determination of OXA at the trace levels in real samples. High enrichment factors (between 40 and 70 for river water) and a high selectivity of the extraction procedure on MIP allow the monitoring of OXA but also for BRO and ALPRA in real samples at very low level of concentration. Indeed, LOQ values in the range 1.2 to 4.5 ng L⁻¹ for river water and from 76 to 357 ng L⁻¹ for urine sample were obtained. Moreover, the clean-up efficiency of the MIP was clearly demonstrated by comparing the results with that obtained with Oasis HLB as a decrease (i) of the complexity of the sample was observed on the LC-MS chromatograms and (ii) of the matrix effects for urine samples.

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethical standards

This is not a clinical study on humans/animals.

Acknowledgment

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SUPPLEMENTARY MATERIAL

Fig. S.1. Chemical structure and octanol-water partition coefficient (log P) of other BZDs included in this study.

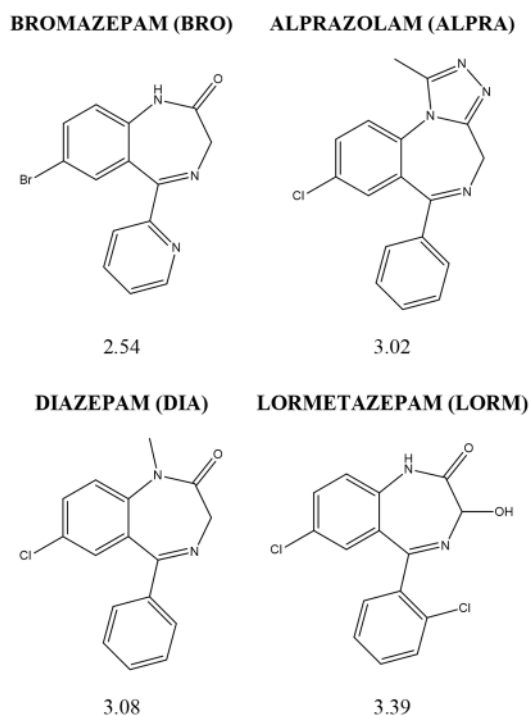


Table S.1. Performances of the LC-UV ($\lambda = 240$ nm) method. The calibration curves consisted of 10 points in a concentration range of 100 to 5000 $\mu\text{g L}^{-1}$.

BZD	Studied linear range ($\mu\text{g L}^{-1}$)	Linear regression	LOD (S/N = 3) ($\mu\text{g L}^{-1}$)	LOQ (S/N = 10) ($\mu\text{g L}^{-1}$)
BRO	100 - 5000	$y = 165 x$ $r^2 = 0.9985$	15	50
OXA	200 - 5000	$y = 122 x$ $r^2 = 0.9977$	33	110
ALPRA	400 - 5000	$y = 84 x$ $r^2 = 0.9791$	90	290
NOR	200 - 5000	$y = 205 x$ $r^2 = 0.9983$	35	120
LORM	200 - 5000	$y = 176 x$ $r^2 = 0.9983$	35	110
DIA	200 - 5000	$y = 97 x$ $r^2 = 0.9973$	55	190

Table S.2. Performances of the LC-MS/MS method. Calibration curves consisted of 11 points in the concentration range from 0.1 to 500 $\mu\text{g L}^{-1}$.

^a Ratio between the area of the peak corresponding to the MRM transition of the quantifier and the area of the peak corresponding to the MRM transition of the qualifier

BZD	t_r (min)	Tube lens (V)	MRM transitions (collision energy, V)		(Area ion quantifier/Area ion qualifier ^a) \pm SD	Studied linear range ($\mu\text{g L}^{-1}$)	Linear regression	LOD (S/N = 3) ($\mu\text{g L}^{-1}$)	LOQ (S/N = 10) ($\mu\text{g L}^{-1}$)
			Quantifier	Qualifier					
BRO	4.5	118	316.1 \rightarrow 182.1 (20)	316.1 \rightarrow 209.2 (16)	1.52 ± 0.10	0.1 - 500	$y = 16131 x$ $r^2 = 0.9976$	0.003	0.01
OXA	8.4	117	287.1 \rightarrow 241.1 (20)	287.1 \rightarrow 269.1 (14)	2.41 ± 0.08	0.1 - 500	$y = 93909 x$ $r^2 = 0.9978$	0.02	0.05
ALPRA	9.5	106	309.1 \rightarrow 281.2 (23)	309.1 \rightarrow 205.2 (39)	2.14 ± 0.09	0.1 - 500	$y = 89769 x$ $r^2 = 0.9905$	0.02	0.05
NOR	12.3	108	289.1 \rightarrow 140.1 (28)	289.1 \rightarrow 226.2 (29)	1.98 ± 0.08	0.5 - 500	$y = 35606 x$ $r^2 = 0.9955$	0.07	0.22
LORM	17.0	108	335.1 \rightarrow 289.2 (21)	335.1 \rightarrow 177.1 (40)	3.04 ± 0.10	0.5 - 500	$y = 199118 x$ $r^2 = 0.9971$	0.06	0.21
DIA	19.4	119	285.1 \rightarrow 193.2 (28)	285.1 \rightarrow 151.4 (24)	2.20 ± 0.06	2.5 - 500	$y = 105967 x$ $r^2 = 0.9958$	0.33	1.1

Table S.3. Comparison between already published analytical methods for the quantification of BZDs in environmental water samples and urine samples and the performance of the developed method in this work.

Number of studied BZDs	Sorbent used for SPE	Type of sample	Percolated volume (mL)	Extraction recoveries (%)	LOQ (ng L^{-1})	Ref.
Environmental applications						
23 ^a	Oasis HLB	Effluent	200	88 - 122	0.1 - 13.2	[13]
2	MIP	River water	100	6, 68	12, 14	[21]
2	Oasis HLB	River water	100	99, 103	0.9, 9.1	[21]
4 ^a	HLB SPE Disk	Effluent	500	77.4 - 104.3	6 - 55	[39]

4 ^a	Oasis HLB	Surface and groundwater	500	83 - 106	10	[40]
7 ^a	Oasis MCX	River water	500	21 - 93	0.5, 1.0	[12]
7 ^a	Oasis MCX	Effluent	100	10 - 110	3.0 - 20.9	[12]
7 ^a	Oasis MCX	River water	200	93 - 114	0.192 - 1.1 (LOD)	[18]
3 ^a	MIP	River water	35	61 - 111	1.2 - 4.5	This work
Applications to urine samples						
4	DSC-18	Urine	ns	81 - 115	ns	[38]
4 ^a	Oasis HLB	Urine	0.1 - 0.2	94.5 - 100.7	ns	[19]
4 ^a	Absolut Nexus	Urine	0.1 - 0.2	94.8 - 101.0	ns	[19]
4 ^a	Bond Elut C18	Urine	0.2 - 0.4	83.5 - 94.5	ns	[19]
6 ^a	Oasis HLB	Urine	2	65 - 122	500 - 5000	[35]
2	MIP	Urine	5	87.2 - 90.4	21500 - 24500	[25]
3 ^a	MIP	Urine	4	38 - 126	76 - 357	This work

^aOXA was among of the targeted BZDs ns: not specified.