# Separation and determination of amoxicillin in wastewater samples using molecularly imprinted polymer nanoparticles followed by HPLC-UV

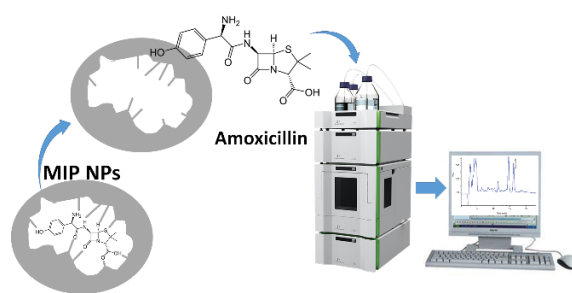Negar Noori, Nazir Fattahi*

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## Abstract

**Molecularly imprinted polymer nanoparticles (MIP NPs) were synthesized using a noncovalent molecular imprinting approach for the selective extraction of amoxicillin from wastewater samples. Solid-phase extraction (SPE) method based on the synthesized MIP NPs followed by high-performance liquid chromatography (HPLC) was used to avaluate the affinity of MIP NPs to amoxicillin. The effect of significant parameters on the extraction process such as sample solution flow rate, breakthrough volume, sample pH, type and volume of the elution solvent as well as the salt addition were investigated and optimized . Under the optimum conditions, the calibration graphs were linear in the range of 0.06–60 µg L$^{-1}$ with a limit of detection (LOD) of 0.02 µg L$^{-1}$. The relative standard deviation (RSD, for 1.0 µg L$^{-1}$ of amoxicillin in wastewater) was 4.1% (n=7). The SPEusing the MIP NPs provided a high enrichment factor (1667) for amoxicillin. These data indicated that the MIP NPs had a perfect selectivity and affinity for amoxicillin and could be used for selective extraction and analysis of amoxicillin in wastewaters.**

## Introduction

In recent years, the presence and fate of active pharmaceutical compounds, including antibiotics, in the environment especially water resources have been recognized as one of the emerging issues in environmental chemistry.[1] In the last 30 years, drug compounds have been considered as the most important water contaminants due to their high variation, high consumption and persistence in the environment.[2,3] Antibiotics account for about 15% of all medications.[4] Amoxicillin is one of the antibiotics belonging to the penicillin group, which is used in medicine and veterinary for the treatment of gastrointestinal and systemic bacterial infections. This compound is resilient against biodegradation and does not easily dissolve in aqueous and soil environments. In the filtration process, amoxicillin does not adsorb solids and its precipitation is very low., after entering of this compound to the soil, in spite of its mobility in soil it dose not evaporated due to its very low vapor pressure. Thus, similar to other antibiotics, this compound is stable in the environment and impose irreparable effects on human health and ecosystems.[5] In countries with no sewage collection networks, sewage discharges from hospitals and health centers will be untreated or incompletely treated and lead to unavoidable hazards to public health.[6]

A variety of analytical approaches have been proposed for the trace-level analysis of antibiotics and their metabolites in different matrices, among which, high-performance liquid chromatography (HPLC)[7-9] and capillary gas chromatography (GC)[10, 11] are of more practical interest. HPLC methods are generally preferred over GC ones, because HPLC can be used without derivatization. Although, it is possible to detect small amounts of antibiotics and their metabolites using this powerful analytical instrument, but it is difficult to determine these analytes at very low concentrations due to matrix interference. Therefore, in order to enhance the sensitivity of this method, there is often a need for a preliminary phase separation and pre-concentration of the samples. In addition, complex samples like wastewater samples are also required to be converted into a form compatible with these instruments.

Many sample pretreatment methods based on sample traits have been developed for the measurement of drugs, antibiotics and their metabolites in trace levels. The frequently used methods are liquid–liquid extraction (LLE),[12] solid phase extraction (SPE),[13] solid–phase microextraction,[14] cloud point extraction (CPE),[15] dispersive liquid–liquid microextraction (DLLME),[16-18] and dispersive liquid–liquid microextraction based on solidification of a floating organic drop (DLLME–SFO).[19-21] LLE and SPE are time-consuming and expensive, while LLE method requires high volume of potentially toxic organic solvents, which is hazardous to health. CPE can also be relatively expensive and often requires a second reagent.[22] In DLLME, the main drawback is the choice of the extraction solvent. Sor this method solvents with densities higher than water are required

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that are not often compatible with reverse phase HPLC. In addition, the high-density extraction solvents, being mostly halogenated, are generally hazardous to laboratory personnel and the environment.[23]

In recent years, Molecularly Imprinted Polymers (MIPs) have been increasingly recognized as a useful tool for studying molecular recognition processes and in the development of sensing systems due to their remarkable selectivity and affinity.[24] Because molecularly imprinted polymers have the properties of special selectivity, easy preparation, simple operation and low solvent consumption, MIPs can replace of the traditional chromatography stationary phases for the purification of active ingredients.[25]

This study was aimed to prepare MIP NPs for amoxicillin, optimize polymerization condition, evaluate binding properties and applications of polymer as the sorbent in SPE, and prepare a sample of amoxicillin in wastewater.

## Experimental

### Reagents and standards

Methacrylic acid (MAA), ethylene glycol dimethacrylate and amoxicillin trihydrate (AMO) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 2,2′-Azobis-iso-butyronitrile was obtained from Acros (Geel, Belgium). Stock standard solution of AMO was prepared in methanol (10.0 mL) with concentration level of 1000 mg $L^{-1}$. Working solution was obtained by appropriate dilution of the stock standard solution daily. The ultra-pure water (six times distillated) used was purchased from Shahid Ghazi Pharmaceutical Co. (Tabriz, Iran). Other solvents and reagents were obtained from Merk (Darmstadt, Germany).

The urban sewage, hospital sewage and river water samples were collected from Kermanshah (Iran) in glass bottles, stored in the dark at 4 °C and analyzed within 24 h of collection. The samples were filtered off through filter paper (Whatman No. 42) before analysis.

### Instrumentation

Chromatographic separations were carried out on a HPLC Knauer with Chromgate software version 3.1 having binary pumps Smartline-1000-1 and Smartline-1000-2 and detector Smartline-UV-2500 variable wavelength programmable (Berlin, Germany), an on-line solvent vacuum degasser and manual sample injector fitted with a 20 μL injection loop (model 7725i, Rheodyne, Cotati, CA, USA). Separations were carried out on a H5-ODS C18 column (25 cm × 4.6 mm, with 5 μm particle size) from Anachem (Luton, UK). A mixture of water–acetonitrile–phosphoric acid–triethylamine (50:49.5:0.25:0.25 v/v) at a flow-rate of 1.2 mL $min^{-1}$ was used as a mobile phase in isocratic elution mode and the detection was performed at the wavelength of 225 nm. The pH values were measured with a Metrohm pH meter (Model: 692, Herisau, Switzerland) supplied with a glass-combined electrode.

### Preparation of MIP NPs

A noncovalent approach was used for preparation of MIP NPs. Amoxicillin as the template and MAA as the functional monomer were dissolved in 12 mL of methanol in a screw-capped glass test tube and incubated at 5°C for 60 min. Then, ethylene glycol dimethacrylate as the cross-linker, and 2,2′-azobis-iso-butyronitrile as the initiator were added. The mixture was sparged, in an ice bath, for 10 min and heated at 50°C for 24 h to complete polymerization. The resultant bulk rigid polymers were crushed, ground into powder, and sieved through a 200-mesh stainless-steel sieve. The polymer particles were washed with methanol/acetic acid (75:25, v/v), centrifuged at 4000 rpm for 5 min, and the supernatant was analyzed by HPLC. Washing was continued until no amoxicillin or any other compound was detected in the supernatant. Blank nonimprinted polymers (NIPs) were prepared in the absence of amoxicillin under the same condition described above.

### Extraction procedure

Forty milligrams of polymer (MIP or NIP), in 3 mL acetonitrile, was slurry packed into an empty polypropylene SPE cartridge. The SPE cartridge was preconditioned with 4.0 mL of acetone, water and water at pH 3.0, respectively. The wastewater samples (100 mL), containing 1.00 μg/L of amoxicillin, were acidified with hydrochloric acid (37%) to pH 4.0 passed through the column at a flow rate of about 10 mL $min^{-1}$ with the aid of a vacuum pump (Rotavac, Heidolph, Germany). The cartridges were rinsed with 5 mL of water at pH 3.0 to remove the matrix interferences. After drying the solid phase by passing air through it for several minutes, the amoxicillin were subsequently eluted with 1.00 mL acetone and were collected into the 10-mL screw cap glass test tubes with conical bottom. The collected phase was evaporated under a gentle nitrogen flow. The residue was dissolved in 30 μL of mobile phase for further HPLC analysis.

## Results and discussion

### Effect of flow rate

Two important factors for the quantitative recovery and desorption of amoxicillin on the SPE cartridge studies are flow rates of the sample solution and elution solvent. The flow rate of the sample solution through SPE controls the analytical time and affects the effective retention of the amoxicillin. The flow rate of the sample solution must be high enough to shorten the analytical time and also, must be slow enough to perform an effective retention to amoxicillin into the adsorbent. The effect of flow rate on recovery of amoxicillin was investigated in the flow rate range of 3–30 mL $min^{-1}$. As can be seen from Figure 1, it was found that in the range of 3–25 mL $min^{-1}$, the amoxicillin recovery by the cartridge was not affected considerably by the sample solution flow rate. As a result, 20 mL $min^{-1}$ was used as the optimized sample flow rate.
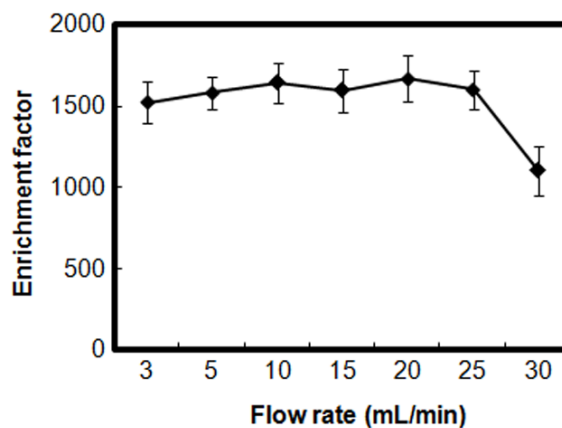


**Figure 1.** The effect of the flow rate on the EF of amoxicillin from SPE NPs. Extraction conditions: water sample volume, 100 mL; eluent solvent (acetone) volume, 1.00 mL; sample solution pH, 4; room temperature.
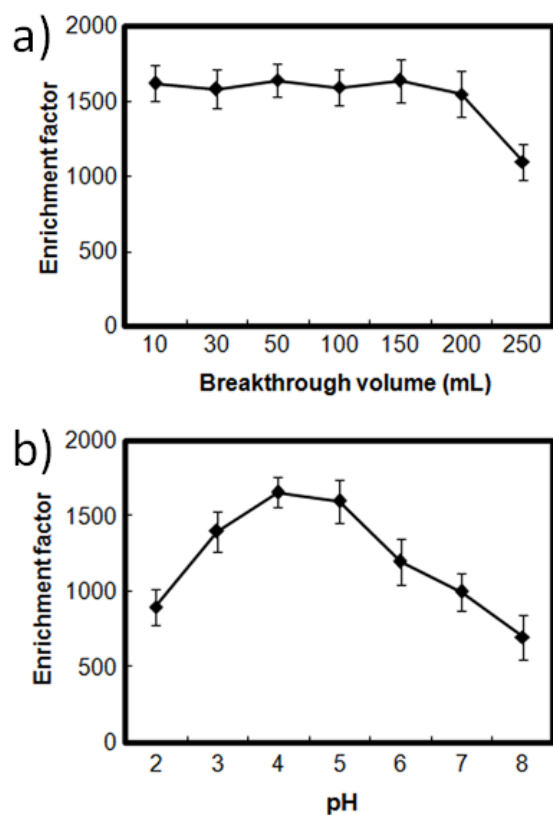
**Figure 2.** *a) The influence of the breakthrough volume on the EF of amoxicillin from SPE nanoparticles. b) The effect of the sample solution pH on the EF of amoxicillin from SPE nanoparticles. Extraction conditions: as in Figure 1; sample solution flow rate, 20 mL min$^{-1}$.*

The flow rate of elution solvent was investigated and quantitative desorption of amoxicillin from the cartridge was achieved in a flow rate of 1 mL min$^{-1}$, using 1.0 ml of acetone. At higher flow rates, quantitative desorption of analytes needed larger volumes of acetone. Therefore, a flow rate of 1 mL min$^{-1}$ was chosen for further studies.

*Effect of the breakthrough volume*

In order to study the influence of the breakthrough volume on the recovery of amoxicillin from wastewater samples, different volumes of sample solution from 10 to 250 mL were passed from the cartridge and then extraction was performed as mentioned in the experimental section. The results in Figure 2a indicate that the recovery remained constant with the breakthrough volume increase from 10 to 200 mL, but then at higher sample volumes, not only was the recovery not satisfactory, but also the analysis time was too long. In order to shorten the analysis time, a sample volume of 100 mL was selected for the following tests.

*Influence of the sample solution pH*

The pH value plays an important role in adsorption of amoxicillin on the cartridge. The effect of pH on the extraction of amoxicillin from wastewater samples was studied in the pH range of 2–8. The higher and lower pH values were not studied because solid phase in this pH values is not resistant. The obtained results in Figure 2b indicated that maximum extraction efficiencies were obtained at pH values around 4 to 5. In more acidic solution, a decrease in the extraction recovery was observed, due to hydrolysis of hydroxide group of the amoxicillin. In addition, considering the acidic constant

of amoxicillin, it is in ionic forms at higher pH and cannot be extracted using the organic solvents. According to the results, pH of 4 was chosen as the optimum pH for extraction.

*Salt addition*

Salt addition is frequently used to adjust the ionic strength, improve the extraction efficiency and reduce the detection limit. Depending on the nature of the target analytes, addition of salt to the sample solution can decrease the solubility of the analytes and therefore enhance extraction because of the salting-out effect. To study the ionic strength effect, the experiments were conducted at different sodium chloride concentrations of the sample solution, ranging from 0 to 5% (w/v). The enrichment factors for amoxicillin obtained highest values when 1% of sodium chloride was added to the samples. Further addition of sodium chloride did not result in an increase in extraction efficiency. Therefore, subsequent experiments were carried out with adding 1% (w/v) salt.

*Influence of the elution solvent type and volume*

In this research, acetone, acetonitrile, and methanol as elution solvent were investigated. The solid phase was eluted using 1.00 mL of each elution solvent. The results illustrated in Figure 3 indicate that the enrichment factor by using acetone, acetonitrile and methanol as elution solvent were 1667, 1610 and 1580, respectively. According to the obtained results and their standard deviations, variations of enrichment factor using different elution solvents are not remarkable, thus, acetone is selected because of low toxicity and cost. For the evaluation of the required acetone volume to elute the amoxicillin from the solid phase, the elution was carried out three times with 1.00 mL of acetone. It was concluded that a volume of 1.00 mL was sufficient to desorb the trapped amoxicillin from the cartridge.

*Quantitative analysis*

The calibration curves obtained under optimized conditions are summarized in Table 1. Linearity was observed in the range 0.06–60 μg L$^{-1}$ with a correlation coefficient (r2) of 0.9990. The precisions were studied by extracting the spiked wastewater sample at the concentration of 1.00 μg L$^{-1}$ for amoxicillin. The relative standard deviations (RSD) was calculated to be 4.1% (n=7). The limit of detection (LOD), based on signal-to-noise ratio (S/N) of three was 0.02 μg L$^{-1}$. The enrichment factor and recovery of amoxicillin were 1667 and 50.3%, respectively.
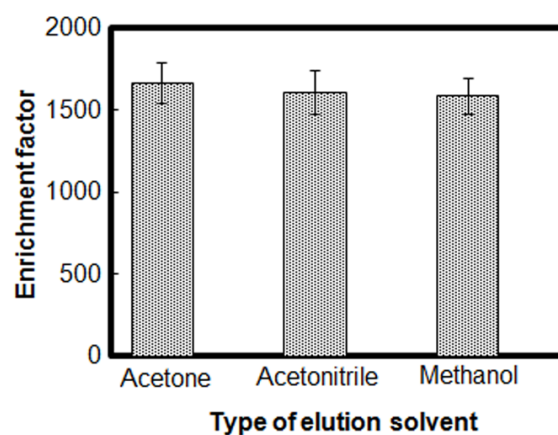


**Figure 3.** *EF of different elution solvents evaluated for extraction of amoxicillin by SPE nanoparticles. Extraction conditions: as in Figure 1; sample solution flow rate, 20 mL min$^{-1}$.*

**Table 1.** *Quantitative results of SPE and HPLC–UV for determination of amoxicillin in wastewater.*

| Parameter | Analytical feature |
|---|---|
| Linear range (µg L$^{-1}$) | 0.06–60 |
| R2 | 0.9990 |
| Limit of detection (µg L$^{-1}$) | 0.02 |
| RSD% (n=7) | 4.1 |
| Enrichment factor | 1667 |
| Extraction recovery | 50.3 |

**Table 2.** *Relative recoveries and standard deviations of amoxicillin from spiked hospital sewage, urban sewage and river water samples.*

| Sample | Added (µg L$^{-1}$) | Found, Mean ± SD$^a$ (µg L$^{-1}$) | Recovery (%) |
|---|---|---|---|
| Hospital sewage | - | 44.2 | - |
| | 5 | 48.9 | 94 |
| | 10 | 53.8 | 96 |
| Urban sewage | - | 11.6 | - |
| | 15 | 27.3 | 104.6 |
| | 20 | 31.1 | 97.5 |
| River water | - | n.d.$^b$ | - |
| | 25 | 23.4 | 93.6 |
| | 30 | 31.5 | 105 |

$^a$SD: standard deviation (n=3).
$^b$n.d.: not detected.
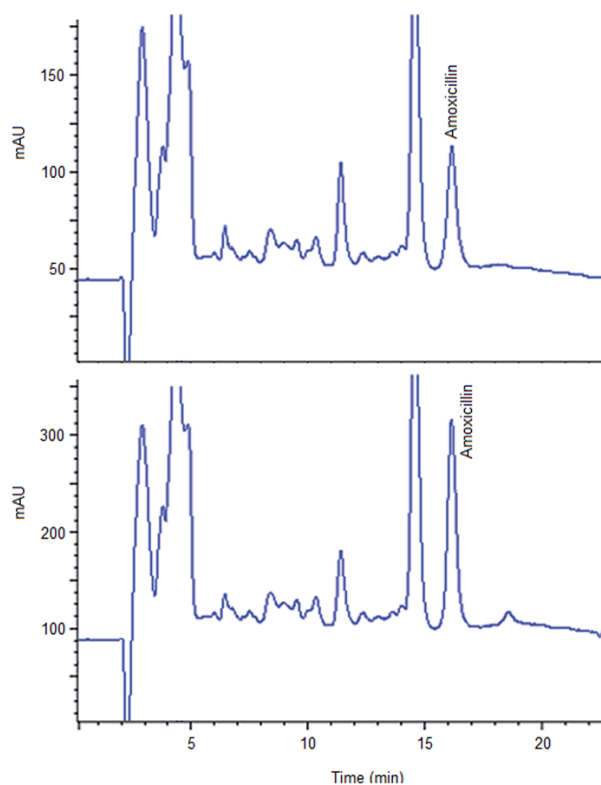
### Real samples analysis

The proposed SPE–HPLC–UV methodology was applied to the determination of amoxicillin in several wastewater samples. Hospital sewage was collected from Imam Reza Hospital (Kermanshah, Iran), urban sewage was collected from Kermanshah and river water was collected from Gharaso River (Kermanshah, Iran). The result for river water showed that it was free of amoxicilline contamination. In the hospital and urban sewage samples, amoxicillin was detected and it was confirmed by spiking amoxicillin into these samples. The concentration of amoxicillin in the hospital and urban sewage samples are 44.2 and 11.6 µg L$^{-1}$, respectively (Table 2). The accuracy of the method was verified by the analysis of the samples spiked with different levels of amoxicillin. The resulted relative recoveries are between 93.6 and 105.0%, which indicates that matrix had little effect on the extraction efficiency. Figure 4 shows the obtained chromatograms of urban sewage and spiked ones at the concentration level of 10 µg L$^{-1}$ for amoxicillin.

### Comparison of SPE–DLLME with other methods

This proposed SPE method based on MIP NPs were compared with other published methods for determination of different antibiotics and drugs. The respective limit of detection (LOD), relative standard deviation (RSD), linear range (LR) and enrichment factor (EF) of each method are summarized in Table 3. The LODs values in SPE nanoparticles were low and the linear range was relatively good. As can be seen, the RSDs of SPE nanoparticles are similar to other methods. Therefore, SPE method based on MIP NPs combined with HPLC–UV is a very simple and sensitive method for the extraction and determination of amoxicillin in real wastewater samples.



**Figure 4.** *The chromatograms of the urban sewage (top) and spiked ones at the concentration level of 10 µg/L for amoxicillin (bottom), obtained using SPE nanoparticles combined with HPLC–UV.*

### Conclusions

In our study, an imprinted MIP for amoxicillin was synthesized by a noncovalent molecular imprinting approach. The MIP was then applied in a SPE protocol, which provides selective extraction of amoxicillin from wastewater samples even at low concentrations. It has been demonstrated that the polymer binds to amoxicillin in wastewater samples as well as samples where the concentration of amoxicillin is considerably low. Amoxicillin recoveries were in the range of 93.6 to 105. The LOD for wastewater sample was 0.02 µg L$^{-1}$. The results indicated that our SPE method based on these MIP NPs could be successfully applied to trace level determination of amoxicillin in wastewater.

### Acknowledgements

**Table 3.** *Comparison of SPE nanoparticles and HPLC–UV with other extraction methods for determination of different antibiotics and drugs.*

| Extraction technique | Analyte | Linear range (µg L$^{-1}$) | LOD (µg L$^{-1}$) | RSD (%) | EF | Reference |
|---|---|---|---|---|---|---|
| HF–LPME– HPLC–UV | Tetracycline | 0.5–1000 | 0.5–1 | 4.3–8.9 | 125–180 | 26 |
| SPE–UHPLC–MS/MS | Glycopeptide | 1–20 | 2 | 1–6.8 | – | 13 |
| DLLME–HPLC–UV | Opium alkaloids | 0.5–500 | 0.2–10 | 2.8–6.1 | 63–104.5 | 16 |
| DLLME–SFO–HPLC–UV | Amphetamines | 10–3000 | 2–8 | 6.2–7.8 | 117–125 | 20 |
| CCSHLLE–DLLME–SFO–HPLC–UV | Amphetamines | 1–3000 | 0.5–2 | 4–5 | 157–168 | 21 |
| SPE–HPLC–UV | Amoxicillin | 0.06–60 | 0.02 | 4.1 | 1667 | This work |

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