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Article

Determination of Cephalosporins by UHPLC-DAD Using Molecularly Imprinted Polymers

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Abstract

Molecularly imprinted polymers (MIPs) were synthesized for the determination of cephalosporins (cephazolin, ceftazidime, cefotaxime, ceftriaxone, cefepime and cephalexin) by ultra-high performance liquid chromatography with diode-array detection (UHPLC-DAD). After optimization, MIPs were synthesized using cephazolin as the template, methacrylic acid as the functional monomer, triethylenglycol dimethacrylate as the crosslinker, acetonitrile/dimethylsulfoxide as porogens and benzoyl peroxide as the radical initiator. Not only this is a novel route of MIP synthesis for cephalosporins, but also this choice of analytes is unique. Chromatographic separation was performed in a C_8 column using a binary gradient with trifluoroacetic acid 0.1% in water and acetonitrile. Linearity was assessed up to 100 μg mL^{-1} and linear correlation coefficients (r) were all higher than 0.99, limits of detection were within the range of 3–12 ng mL^{-1}, and recoveries from 86 to 102% were obtained for concentrations between 0.05 and 1.0 μg mL^{-1}.

Introduction

Cephalosporins are structurally and pharmacologically related to penicillin. Like them, cephalosporins have a β-lactam ring structure that interferes with the synthesis of the bacterial cell wall (1, 2). They are used for the treatment of infections caused by Gram (+) and Gram (−) bacteria. They are among the safest and most effective broad-spectrum bactericidal antimicrobial agents and, consequently, they are the most frequently prescribed class of antibiotics (3, 4).

Cephalosporins are recognized as environmental emerging contaminants and are included in the group of pharmaceutically active compounds, although no indicative tolerable values for these substances have yet been fixed. Of course, one of the main concerns associated with their use is the development of resistances. This has already been detected in waste water and sewage treatment plants as well as in other environmental compartments (5–7). This situation is especially critical in developing countries where the high rates of self-medication associated with the direct discharge of untreated sewage into the environment strongly affects the water quality (8–10).

However, for environmental applications, a pre-concentration and cleaning step is needed prior to determination (11), this is usually complex and difficult in the case of cephalosporins due to their high solubility and low retention during conventional solid phase extraction (SPE). In this context, molecularly imprinted polymers (MIPs) can be a useful tool to solve these problems allowing to

reach very low levels when used as sorbents in SPE with a high degree of selectivity while in complex samples (12–18). Although there have been prior works developing MIPs for cephalosporins (15, 19–21), it is still a relevant research field where new routes of synthesis can be experimented. To the best of our knowledge, it is not only the first time this particular list of chephalosporins is analyzed but also the first time this combination of reagents is used to synthesize MIPs. Moreover, the use of ultra-high-pressure liquid chromatography (UHPLC) was particularly suited for rapid method development and optimization (22).

Materials and Methods

Chemicals

Acetonitrile (ACN) and methanol (MeOH) (HPLC grade), phosphoric acid (85% *w/v*), acetone (99.7% w/v), dimethylsulfoxide (DMSO, 99.5% w/v), hydrochloric acid (32%) were all purchased from Uni-Chem (China). Trifluoroacetic (TFA > 98%) and acetic acid (99.8%), hidroxiethyl methacrylate (HEMA, 95%), triethylenglycol dimethacrylate (TEGDMA) (95%), benzoyl peroxide (70%), methacrylic acid (MAA, 99.5%), tetrabuthylammonium hydroxide (TBA-OH, 0.1 mol L^{-1} in propan-2-ol/methanol 10:1 (*v/v*)), acetone (99.8%) and dimethylsulfoxide (DMSO, ≥99%) were all purchased from Merck (Germany). Ceftazidime (pentahydrate, AZI), cephazolin (sodium salt, AZO), cefepime (hydrochloride, EPI), cefotaxime (sodium salt, TAX), ceftriaxone (sodium salt, AXO) and cephalexin (hydrate, ALE) were all USP standards.

All aqueous solutions were prepared using ultrapure water with resistivity not <18.2 MΩ cm at 298 K (Heal Force, China).

Chromatographic separation

UHPLC with diode-array detection (UHPLC-DAD) was performed with a chromatograph from Shimadzu (Japan) with two pumps LC-0AT, auto-sampler SIL-20A, oven CTO-20A, SPD-M20A detector, and with software LC Solution v. 1.24. A mixture of trifluoroacetic acid 0.1% in water (A) and ACN (B) was used in a gradient way as the mobile phase. The binary gradient followed this scheme: (i) 0–3 min: 90% (A), 10% (B) isocratic; (ii) 3–5 min: 82% (A), 18% (B); and (iii) 5–20 min: 82% (A), 18% (B) isocratic. Spectrophometric detection was performed at 264 nm.

The column was chosen between four different options (C1–C4), all of them with 250 mm of length, 4.6 mm of internal diameter and 100 Å pore size, their specifications were the following: (C1) Alimatic (Sphermatic, Spain), C_8, 5 µm; (C2) Daisogel (Shimadzu, Japan), C_{18}, 10 µm; (C3) Shimpack (Shimadzu, Japan), C_8, 5 µm; and (C4) Kromasil (Phenomenex, USA) C_8, 5 µm.

MIPs' synthesis

AZO in its molecular form was obtained by adding 350 mg of AZO (sodium salt) to a beaker with 50 mL purified water shaking until complete dissolution. Then, 5 mL of phosphoric acid (20%) were added. The precipitated was then filtered, washed three times with 50 mL of purified water and dried in an oven for 48 h at 40°C. In a flask of 5 mL, 100 mg of the mentioned precipitate, 500 µL of ACN and 120 µL of DMSO were all mixed, the latter was necessary to obtain a complete dissolution of AZO. Afterwards, 85 µL of HEMA were added mixing carefully and letting the mixture rest for one hour in the dark. Then, 1,278 µL of TEGDMA and 20 mg of benzoyl peroxide were incorporated, shaking and bubbling Helium during 3 min. Finally, the reaction mixture was heated for 24 h at 60°C. This procedure was applied in synthesis of M1, for the others MIPs' synthesis (M2, M3, M4, M5, M6) a similar procedure was applied, but varying the porogens and monomers according to Table I, maintaining molar proportions at 1:4:20 (template: monomer: crosslinker) (23–25). With M2 and M5, 500 µL of DMSO were used instead of 120 µL. M4, M5 and M6 were synthesized in the same way but using MAA 74 mg, as functional monomer. NIPs' preparation were made by using the same reagents and porogens but without the addition of any template.

Table I. Reagents Used in the Different MIPs

	M1	M2	M3	M4	M5	M6
Template	AZO					
Monomer	HEMA				MAA	
Crosslinker	TEGDMA					
Porogen 1	ACN	–	acetone	ACN	–	acetone
Porogen 2	DMSO					
Initiator	benzoyl peroxide					

Both group of polymers, MIPs and NIPs, were grounded in a mortar, sieving and recovering the fraction of 36–71 µm. Then they are washed in three stages, first with 500 mL of MeOH (5 × 100 mL), 300 mL of TFA 1% (3 × 100 mL) and finally with 200 mL of diethyl ether (2 × 100 mL). A monitoring step before they were used was carried out in every stage to confirm the absence of templates or related products of degradation after synthesis. Finally, the polymers were dried at 60°C for 24 h and kept in dry cleaned flasks until use.

Rebinding studies

The 40 mg of each MIP/NIP were weighted in 2 mL flasks, and 1.0 mL of a standard solution of 1 µg mL^{-1} of the mixture of the six cephalosporins was added, and then shaken for 8 h. The final concentration in the supernatant was measured by UHPLC-DAD.

Results and Discussion

Optimization of the chromatographic separation

The detection wavelength was selected favoring those cephalosporins with less specific absorbance in a comparison made with a 10 µg mL^{-1} solution of the six compounds. Wavelength selection was carried out by overlapping their UV spectra and maximizing the signal of less absorbent cephalosporins, in this case ALE, EPI and AZO (data not shown). A range from 260 to 268 nm was identified as appropriate for these three cephalosporins. In the end, an average wavelength (264 nm) was selected for all the assays. In order to obtain a suitable chromatographic separation, due to the similarity in the chemical structure of this family of antibiotics, a binary gradient was employed. The ionic suppression of carboxylic groups occurred due to higher acidity of TFA ($pK_a = 0.6$) increasing the interaction with the stationary phase due to less solubility of the protonated form of cephalosporins versus their ionic ones. Moreover, the use of ACN as an organic modifier allowed to elute them according to their solubility in the mobile phase when the amount of ACN was changing with time as expected in a distribution mechanism. The combination of these two different effects produced an appropriate separation.

Column selection was made by a comparison of the analytical behavior of the several columns (C1–C4, as described in the Materials and Methods) with reverse phase under the proposed

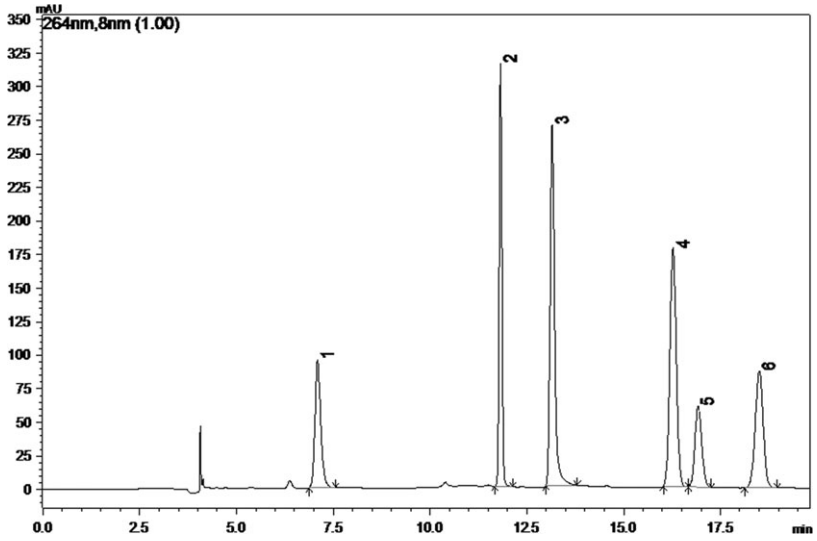

Figure 1. Chromatogram of a standard solution with the six cephalosporins in a concentration of 10 μg mL^{-1}: EPI (1), AZI (2), AXO (3), TAX (4), ALE (5) and AZO (6).

elution program (Table S1 from the Supporting Information). The mobile phase selected separated all compounds in all columns, with exception of TAX and AZO, with a resolution between them of 1.1 units in column 1, while a convenient result would be higher than 1.5, thus column 1 was considered unappropriated to be used. The asymmetry factor (*T*), related with free silanols, evidenced that columns 2 and 3 were not fully adequate, because there is a little peak distortion produced, indicated by *T* values outside the range of 0.95 and 2.0, as it is known that peak asymmetry increases, integration, and hence precision, becomes less reliable, being not adequate to quantify. Finally, column 4 was selected for the final method validation and application. It was with column 4 that the highest theoretical plates' numbers was achieved, which provides information about fast equilibriums between both phases, stationary and mobile, and, therefore, thinner peaks in shape were obtained, this is of great importance for a correct quantification. Under all these conditions, the elution order was EPI (1), AZI (2), AXO (3), TAX (4), ALE (5) and AZO (6), a chromatogram is shown in Figure 1.

Stability studies

Some authors have described a slow degradation of a variety of cephalosporins in water solutions (26), so it is probable to find degradation products over certain period of time, especially in acidic/basic media. Thus, initially, a stability study was carried out in different conditions: water, ACN–water (20–80% in volume), H_3PO_4, 1% in water, all for 10 h. Stability studies were performed with a standard solution of a cephalosporin mixture of 0.2 μg mL^{-1}. The presence of degradation products was considered by monitoring peak purity for each compound. Results demonstrated that in purified water compounds are stable for ~8 h. Same results were obtained with the ACN–water mixture (data not shown). But when the compounds' stability was analyzed in H_3PO_4, some of the cephalosporins were unstable. In this case, EPI, AZI and ALE were stable with an RSD below 2.1, 3.0 and 0.3%, respectively. TAX and AZO showed a moderately linear degradation with a RSD < 7.0 and 15.0%, respectively, while the degradation of AXO was clearly observed, decreasing the signal until was not detected at 5 hours (Figure S1 in Supporting Information) which motivated its elimination for further studies. However, when monitoring the peak of the remaining five cephalosporins none interference, due to the formation of degradation products, was detected.

MIPs' development

It is well known that the porogen plays an important role in recognition, thus usually ACN in pure solutions and mixtures is used because it does not affect hydrogen bonding between templates and monomers. The use of mixtures as porogens is only justified when solubility is compromised, as in this case, where DMSO and acetone act as co-solvents for total dissolution of template–monomer combination. In addition, the use of DMSO as pure solvent allowed to understand how hydrogen bonding or electrostatic interaction is affected when ACN and acetone are used in combination with HEMA in one case and MAA in other. In this study, M2 an M5 and their corresponding N2 and N5 were obtained with the aforementioned purpose. For all the experiments performed an RSD < 9.7% ($n = 3$) was obtained. The use of a single molecule as template for a family of compounds in common in the development of MIPs (27–30). The choice of AZO as template was merely empirical and not based in any theoretical or experimental results, not only AZO is a relevant antibiotic but it also has significant groups in both ends of the cephalosporin's root core (Figure 2).

When recoveries from MIPs and NIPs in water are compared (Figure 3A, an example of a chromatogram is shown as Figure S2 in Supporting Information), a higher rebinding for MIPs is observed in M1, M4 and M6 versus their respective NIPs, which is evidence of a presence of a recognition site. Higher retention of TAX is observed in M4, probably because of a strong interaction among the amino from the five-member ring in the analyte and carboxylic groups (coming from MAA residue after polymerization) located in this polymer (M4) both groups ionized at this pH, favoring electrostatic interaction. Permanent charged nitrogen in AZI and EPI structures explains why their retention are too low, despite of amino group in the same position that in TAX which produces very high solubility and a drain process through the polymer probably by a more efficient dissolution effect. Non-specific interaction cannot be discarded in this case, according to NIP behavior at the same conditions

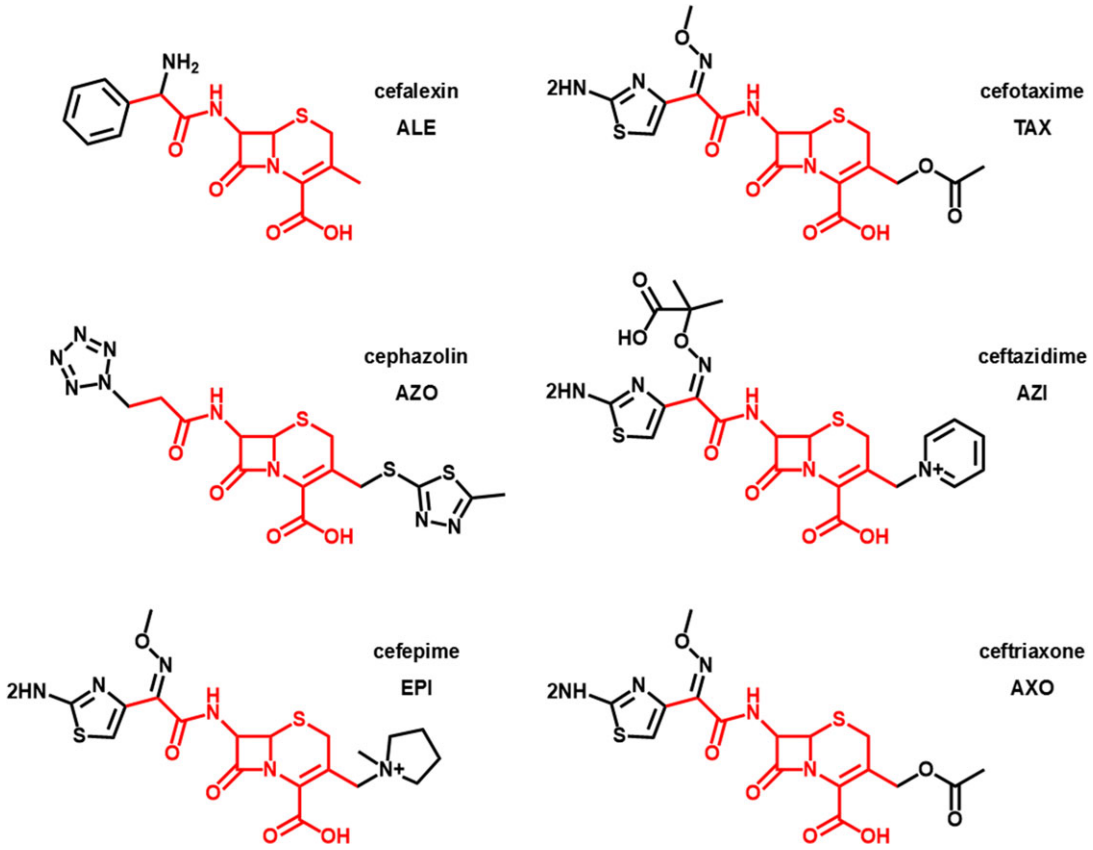

Figure 2. Chemical structure of the cephalosporins studied.

(Figure 3A), thus, the difference in rebinding among them, supports the creation of a complementary cavity with TAI making M4 the most useful for continuing studies. In addition, some differences in rebinding are observed between M1 and N1. Besides the recognition site formation as expected in M1, this behavior can be related by formation of a hydrogen bonding between free OH from the polymer and polar groups in cephalosporins' structure, which are better accommodated in the recognition sites. However, lower recoveries in this study pointed to a competition between the interaction of analytes with the complementary cavity and hydrogen bonding formation with these cephalosporins and water, which seems to be stronger than the previous one causing a draining of the compounds out from imprinted and not imprinted polymers (M1 and N2), leading to lower recoveries when compared to M4. Finally, M6 has the same differences that M1, but interactions must be of dipole–dipole nature between monomer and analyte, which are weaker than the hydrogen bonding formation with water, explain the retention caused by the use of acetone as porogen instead of ACN with M4.

Higher recoveries over 60% in MIPs during rebinding studies in ACN–water 10:90 (*v*/*v*) (Figure 3B) are clearly noticed. ACN allows hydrogen formation which increases analyte-polymer specific and not specific interactions, implicating that selectivity of all polymers are poor using this mixture. Although, as was expected, retention of AZO was higher because it is complementarity with the recognition site in the polymer, thus, a washing stage could be efficient to evaluate in a better way the specificity of MIPs when sample is loading under this condition. However, this behavior supports the existence of a tridimensional cavity highly selective to AZO when the other cephalosporins are taken into account. Van der Waals forces are involved in recognition and retention under this rebinding condition because retention increases as well as octanol/water partition coefficient (also known as log *P*) does for the sequence EPI → AZI → TAX → ALE → AZO (log P: −4.3 → −1.6 → −1.2 → 0.65 → 1.13).

Acidic media (H_3PO_4, 1% in water) (Figure 3C), showed a different scenario. In this medium, non-ionized carboxylic groups are predominant (molecules and polymers with MAA), thus, hydrogen bonding is the only possibility of the main interaction. Three of the five cephalosporins are better retained, in this case EPI, TAX and AZO. Despite of this, a great selectivity was not reached in these conditions, while recoveries >60% were obtained for EPI in MIPs obtained with HEMA (M1, M2 and M3) when compared which those prepared with MAA (M4 and M5). MIP M6 behavior was very much alike to M1, M2 and M3 in rebinding process. TAX was recovered with some selectivity from M1 and M5, meanwhile in others polymers, with similar or even high recoveries, no specificity could be observed. AZO showed selectivity only in M5, although its recoveries were significantly higher than TAX. The same situation previously observed (Figure 3B) is expected to happen related to the presence of a recognition cavity, but in these conditions, the non-specific interactions are promoted due to common hydrogen bonding which affects the selectivity. Results appear to show there is no clear homogeneity in behavior. Electrostatic and Van der Waals interactions as well as hydrogen bonding are some of the aspects to take into account when a MIP for cephalosporins is about to be prepared. Taken all into consideration, M4 seems to be the best fit for future experiments (Figure 4).

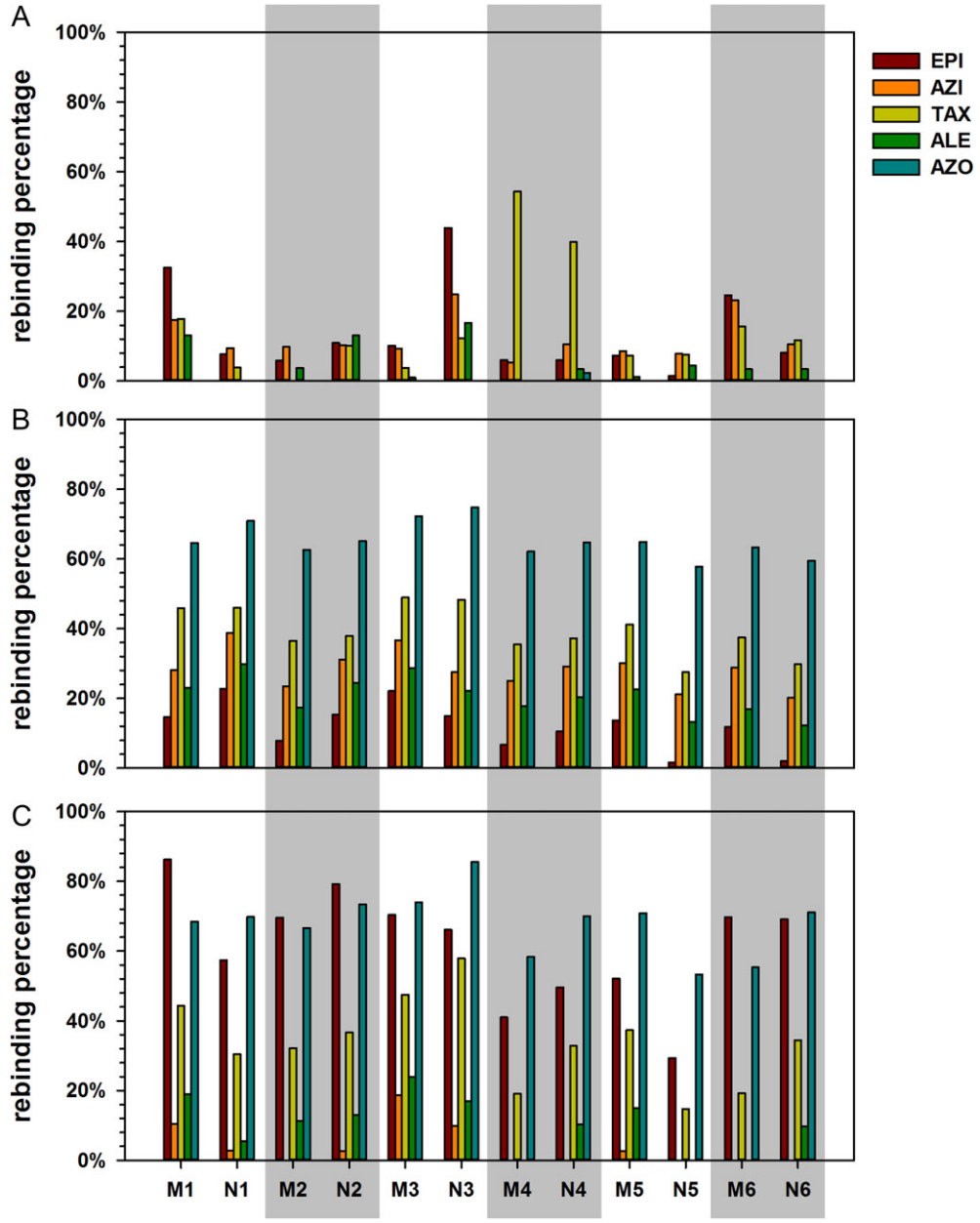

Figure 3. Rebinding studies: 8 h, neutral pH, 25°C, analyte concentration 1 μg mL^{-1}. (A) water; (B) ACN–water mixture 10:90 (v/v); and (C) water with H_3PO_4 1%.

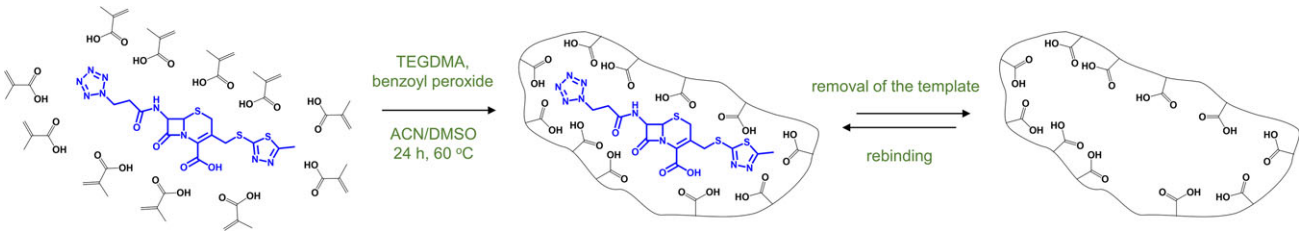

Figure 4. Schematic representation of MIPs formation.

Although there can be found a few works in literature for the determination of cephalosporins with MIPs, namely for ALE (19, 21); ALE and cephapirin (15); cefthiofur, AZO, cefquinome, cephapirin, ALE and cephalonium (20). None of them, however, analyzed these particular six cephalosporins simultaneously. This difference arises from a different procedure of synthesis, in

Table II. Statistic Parameters for Linearity, LOD and LOQ for all the Cephalosporins

Cephalosporin	*R*	RSD (%)	*P*-value	*P*-value	LOD (ng L^{-1})	LOQ (ng L^{-1})
EPI	0.996	9.9	0.68	0.52	7	20
AZI	0.996	9.1	0.82	0.96	3	8
TAX	0.999	3.0	0.95	0.90	6	19
ALE	0.999	4.9	0.86	0.68	12	20
AZO	0.990	9.8	0.68	0.95	9	28

Table III. Precision Results Obtained With the Proposed Methodology

Cephalosporin	Concentration (μg mL^{-1})	RSD intra-day, $n = 6$ (%)	RSD inter-day, $n = 6$ (%)
AZO	0.05	6.7	3.0
	0.10	4.2	11.8
	1.0	4.1	4.1
TAX	0.05	7.6	8.7
	0.10	8.6	2.5
	1.0	1.6	2.6
AZI	0.05	10.3	5.1
	0.10	8.5	1.5
	1.0	0.9	1.2
EPI	0.05	7.3	4.0
	0.10	4.3	4.0
	1.0	1.8	1.4
ALE	0.05	9.1	6.8
	0.10	4.2	1.7
	1.0	3.8	1.7

particular, the use benzoyl peroxide as the radical initiator. Lata *et al.* (19) used 2-(trifluoromethyl) acrylic acid (TFMAA) as the functional monomer, ethylene glycol dimethacrylate (EGDMA) as the crosslinker and azobis-isobutironitrile (AIBN) as the radical initiator. Quesada-Molina *et al.* (15) used MAA as the functional monomer, EGDMA as crosslinker and AIBN as the radical initiator. Baeza *et al.* (20) used *N*-3,5-bis(trifluoromethyl)phenyl-*N*′-4-vinylphenyl urea (VPU) as the functional monomer, divinylbenzene (DVB) and AIBN 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV) as the radical initiator.

Analytical parameters

Linear correlation coefficients (*r*) were higher than 0.99 for all analytes with RSD between 3 and 10%. Durbin Watson Test (*P*-value > 0.05) demonstrated that RSD of response factor is random and no critical error were obtained. Values for the limit of detection (LOD) and quantification (LOQ) were the following: 3–12 ng L^{-1} for LOD and 8–28 ng L^{-1} for LOQ (Table II).

Accuracy, studied on a range from 0.05 to 1.00 μg mL^{-1}, showed a matrix effect in acid medium (H_3PO_4, 1% in water) (data not shown) but values 86.3–101.8% with RSD of 0.02–0.80% could be reached, probably agree with their polar nature that can produce some interaction with sylanols of the glass in which they are collected for the studies, or the decrease in solubility according to a suppression of ionization in carboxylic groups. Possible precipitation was not discarded. Matrix effect was not observed in ACN–water 10:90 (*v/v*). Precision (intra-day and inter-day) showed values of RSD from 0.9 to 12% within the concentration interval from 0.05 to 1.0 μg mL^{-1} (Table III).

Conclusions

MIPs were synthesized for the UHPLC-DAD determination of several cephalosporins using cephazolin as the template. Suitable analytical parameters, namely low LODs, good linearity and suitable recoveries, were obtained by M4 (methacrylic acid as the functional monomer, triethylenglycol dimethacrylate as the crosslinker, ACN/DMSO as porogens and benzoyl peroxide as the radical initiator). This MIP–UHPLC-DAD procedure might be used in the future for cephalosporin analysis in complex samples.

Supplementary Data

Supplementary data are available at *Journal of Chromatographic Science* online.

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Conflict of interest statement. The authors declare no conflict of interest.

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