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Fixed-Bed Column Process as a Strategy for Separation and Purification of Cephamycin C from Fermented Broth

Liliane M. Oliveira,[†] Luciana M. Brites,[†] Maritza C. C. Bustamante,[†] Pier Parpot,[‡] José A. Teixeira,[§] Solange I. Mussatto,[*,‖] and Marlei Barboza[⊥]

[†]Post Graduation Program in Chemical Engineering, Universidade Federal de São Carlos, Rodovia Washington Luiz, km 235, 13565-905 São Carlos, Brazil

[‡]Centre of Chemistry and [§]Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[‖]Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

[⊥]Department of Chemical Engineering, Universidade Federal do Triângulo Mineiro, Avenida Frei Paulino, 30, Bairro Abadia, 38025-180 Uberaba, Brazil

ABSTRACT: Fixed-bed column processes using the anionic resin Q Sepharose XL were evaluated for cephamycin C (CepC) purification from fermentation broth. Breakthrough and desorption curves were obtained for different flow rates (2.5, 5.0, and 7.5 mL/min). The elution method consisted of a stepwise gradient using NaCl solutions (0.1%, 0.3%, and 0.5%), which resulted in the separation of CepC from other antibiotics. The flow rate did not interfere in adsorption during loading of the column, but band broadening was observed during elution as the flow rate was increased. After the ion-exchange process, the fractions containing CepC were subjected to solid-phase extraction using a C18 cartridge to remove salts. Analyses of the broth used to feed the column and of the salt-free fractions by LC−MS showed a reduction in the concentration of some contaminants (possibly penicillin N, deacetylcephalosporin C, and deacetoxycephalosporin C) compared to the concentration of CepC. In conclusion, ion exchange followed by adsorption on a C18 adsorbent was demonstrated to be a selective and efficient procedure for the purification of CepC from fermentation broth.

1. INTRODUCTION

Cephamycin C (CepC) is an important β-lactam compound belonging to the class of cephalosporins. As a β-lactam antibiotic, it affects peptidoglycan synthesis in prokaryotes, affecting cell-wall integrity.[1] In the cephalosporin group, CepC is more active against Gram-negative and anaerobic bacteria than the other cephalosporins, and it is also more resistant to some types of β-lactamase enzymes.[2] These enzymes are produced by some pathogenic bacteria and represent one of the most important mechanisms of bacterial resistance to β-lactam antibiotics.[3]

CepC is usually produced by submerged fermentation with *Streptomyces clavuligerus* and *Nocardia lactamdurans*.[4] To obtain this antibiotic at an adequate purity level, the use of appropriate downstream techniques is essential to isolate it from the fermentation broth. Separation techniques based on adsorption and ion-exchange processes have been widely applied for the isolation and purification of products obtained during bioprocesses.[5−10] These processes stand out from other separation techniques because they provide a high concentration factor, which satisfies the need for a high recovery of the product of interest.[11] Moreover, they are nondenaturing, highly selective, and relatively inexpensive processes.[12] For biopharmaceuticals, ion exchange and adsorption are mainly carried out by packed-bed chromatography. The prevalence of this unit operation is mainly due to the high-resolution separations obtained even for similar compounds. Advances in resin chemistry and synthesis have improved resin selectivity and mechanical resistance, making these materials adequate to satisfy industrial demands for high purity and throughput.[13,14]

Ion exchange can be considered as an interesting technique for the extraction and purification of CepC from fermentation broths, because of the amphoteric nature of this molecule. CepC presents amino and carboxylic groups, which, depending on pH, are ionized and can bind to the active sites of the resin by displacing other previously bonded ions.

Although the discovery of CepC was announced between 1971 and 1972, by researchers from the Lilly and Merck laboratories,[3] literature data about processes for CepC purification are scarce. Most of the available information has been reported in patents, and the most recent patent was published more than 30 years ago, in 1982.[15−19] In the processes claimed by these patents, operations of filtration, ion exchange on polymeric resins, and adsorption on activated carbon or on neutral polymeric resins were employed. Among these patents, that of Kawamura and co-workers claimed a process to obtain the sodium salt of CepC, and in addition to the unit operations already mentioned, the authors also used size-exclusion chromatography, precipitation with acetone, and thin-layer chromatography.[16] After the patent granted to Kamogashira et al.,[15] only the recent study by Baptista-Neto et al. on the purification of CepC from fermentation broth can

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be found in the literature.[5] These authors evaluated the adsorption of CepC in fixed-bed columns using the anionic resin Q Sepharose XL and the neutral resin XAD1180. The results showed that CepC was able to adsorb onto the anionic resin, but that the antibiotic was weakly bound to the neutral resin, being eluted during the column washing step. This study did not show separation of different compounds during elution for any of the resins evaluated.

One of the greatest difficulties hindering the study of CepC production and purification is the absence of a standard compound.[4] As CepC is not directly used as an antibiotic, a standard for this compound is not commercially available. Purification studies of this antibiotic are of great significance, because they can provide useful information on achieving the pure form of CepC. Therefore, in this study, the separation and purification of CepC from fermentation broth was evaluated using the ion-exchange technique in fixed-bed column processes. The resin Q Sepharose XL was used because of its good adsorption capacities, as already reported in the literature.[5] We investigated the influence of different flow rates on the column performance by obtaining breakthrough and desorption curves. To obtain a purified fraction of CepC, an elution method was proposed, and the separation of CepC from contaminants was evaluated. As chemical standards for CepC and the main contaminants were not available, an alternative method for qualitatively evaluating the separation of CepC from contaminants was to use mass spectrometric analysis, which is a technique that is sensitive enough to detect compounds present in very low quantities according to their molecular weight. In this analysis, all of the ionizable compounds present in a sample are detected. Therefore, the signal intensities of the ions generated by the molecules of CepC and contaminants could be compared, and the purification achieved could be evaluated.

2. EXPERIMENTAL SECTION

2.1. Resins. The neutral resin Amberlite XAD4 was purchased from Sigma (Lyon, France). According to the manufacturer's specifications, this resin has the following characteristics: styrene/divinylbenzene matrix, mean pore size of 100 Å, pore volume of approximately 0.98 mL/g; surface area of 750 m^2/g, and particle size of 20−60 mesh (0.25−0.85 mm). Before use in the experiments, XAD4 was sequentially treated with 50% methanol, deionized water, 50% ethanol, and deionized water, in an agitated tank.

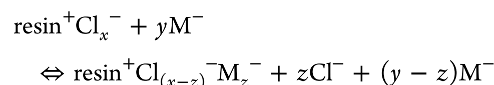
The anionic resin Q Sepharose XL (QXL) was purchased from GE Healthcare (Stockholm, Sweden) and had the following characteristics: matrix structure formed by cross-linked 6% agarose with bound dextran, spherical beads with a mean particle size of 90 μm, and ionic capacity of 0.18−0.25 mmol of Cl^-/mL of adsorbent. The QXL pretreatment was carried out in a column by sequentially passing through the bed three bed volumes (BV) of 0.5 N NaOH + 1 M NaCl, 2 BV of deionized water, 1 BV of 25% acetic acid, and finally, 4−5 BV of deionized water.

2.2. Fermentation Broth and Pretreatment Conditions. CepC was produced by *Streptomyces clavuligerus* (DSM 41826) under batch fermentation conditions, according to the methodology described by Bellão et al.[20] The fermentation medium consisted essentially of soybean derivatives and lysine as nitrogen sources and glycerol as a source of carbon and energy.

Before the ion-exchange experiments, the fermentation broth was subjected to three sequential treatments: microfiltration, ultrafiltration, and adsorption on the resin Amberlite XAD4 for pigment removal. Filtrations were carried out in hollow-fiber cartridges (GE Healthcare, Wauwatosa, WI). Membranes with a pore size of 0.22 μm and 3 kDa and filtration areas of 3600 and 2800 cm^2 were used for microfiltration and ultrafiltration, respectively.

The adsorption process for pigment removal was carried out by pouring the pretreated XAD4 resin and the ultrafiltered broth into a stirred tank, and the system was maintained under agitation for 30 min at room temperature. Then, the resin was separated from the clarified broth, which was used in the ion-exchange experiments. At the end, the pH of the clarified broth was between 6 and 6.5. This operation was necessary to avoid loss of the QXL resin, as colored compounds can irreversibly adsorb onto the resin.[5]

2.3. Fixed-Bed Experiments. As already mentioned, the clarified broth's pH was between 6 and 6.5. The pK_a of CepC is 2.5; therefore, the molecules were negatively charged during the ion exchange. The adsorption onto and desorption from the anionic resin involved an ion-exchange equilibrium according to the following mechanism



$$\text{resin}^+\text{Cl}_x^- + y\text{M}^- \Leftrightarrow \text{resin}^+\text{Cl}_{(x-z)}^-\text{M}_z^- + z\text{Cl}^- + (y-z)\text{M}^-$$

where $\text{resin}^+\text{Cl}_x^-$ represents the anionic resin in the Cl^- form and containing x actives sites; M^- represents the negatively charged molecules in the broth, which could be both CepC and contaminants; and $\text{resin}^+\text{Cl}_{(x-z)}^-\text{M}_z^-$ is the resin with z binding sites occupied by M^-. During column loading, the concentrations of species M^- in the broth were high, and the reaction shifted toward the right. During the elution step, however, the concentration of Cl^- was increased, shifting the system to the left and releasing $CepC^-$ and other M^- compounds.

The column experiments were planned to evaluate the influence of the flow rate on the breakthrough curves and the column elution. A jacket glass column (GE Healthcare, Stockholm, Sweden) with an internal diameter of 1 cm was used, and the bed heights for the assays were 25.6 cm (CR1), 26.0 cm (CR2), and 25.6 cm (CR3). Three flow rates were tested, namely, 2.5 mL min^{-1} (CR1), 5.0 mL min^{-1} (CR2), and 7.5 mL min^{-1} (CR3), corresponding to the linear velocities 5.3, 10.6, and 15.9 m s^{-1}, respectively.

The procedure used for the clarified broth purification consisted of the following steps: (1) application of approximately 50 mL of clarified broth to the column; (2) bed washing with 100 mL (∼5 BV) of deionized water to remove unabsorbed molecules in the interstitial space; (3) elution using a stepwise gradient of 400 mL (∼20BV) of 0.1% NaCl, 400 mL of 0.3% NaCl, and 400 mL of 0.5% NaCl. Fractions of 3 mL (during the feed step) and 20 mL (during the other steps) were collected to determine the CepC concentration, the antimicrobial activity, and the presence of contaminants. These assays were carried out at 20 °C.

During the elution of the column, fractions containing CepC were collected, freeze-dried (FreeZone Freeze-Dry Systems, LABCONCO, Kansas City, MO), and kept at −80 °C for posterior analysis in a mass spectrometer. These analyses were performed to detect ions corresponding to the molecules of CepC and contaminants. The results were used to qualitatively

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evaluate the purification obtained in the ion-exchange column process.

2.3.1. Breakthrough Curves. The breakthrough curves were determined by plotting the changes in the concentration of CepC (expressed as C/C_0) along the experiment, during the feeding step and part of the washing step of the column. C and C_0 are the CepC concentrations in the column outlet and in the feed, respectively. From these curves, some variables related to the resin uptake capacity and bed utilization were calculated, and the process efficiency was determined. The variables calculated were the breakthrough time (t_b), the amount of CepC adsorbed per bed volume until column saturation (q_t) (eq 1), the column bed volume (BV) (eq 2), the bed utilization (ϕ_1) (eq 3) and product recovery (ϕ_2) (eq 4) efficiencies, and the productivity (P) (eq 5). The calculations were carried out according to previously described methodologies[21,22] using the equations

$$q_t = \frac{C_0 Q}{\mathrm{BV}} \int_0^{t_e} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t \tag{1}$$

$$\mathrm{BV} = \frac{\pi D^2}{4} H \tag{2}$$

$$\phi_1 = \frac{\int_0^{t_b} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t}{\int_0^{t_e} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t} \tag{3}$$

$$\phi_2 = \frac{\int_0^{t_b} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t}{\int_0^{t_e} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t} \tag{4}$$

$$P = \frac{\int_0^{t_e} \left(1 - \frac{C}{C_0}\right) \mathrm{d}t}{t_e} C_0 Q \tag{5}$$

where C_0 is the CepC concentration in the clarified broth, C is the CepC concentration at time t, Q is the flow rate, BV is the column bed volume, t_e is the time at which $C/C_0 = 1$, and t_b is taken as the time at which $C/C_0 \approx 0.1$.

2.3.2. Desorption Analysis. The influence of flow rate during the desorption step was evaluated according to the van Deemter equation, which relates the height equivalent to a theoretical plate (HETP) and the linear velocity

$$\mathrm{HETP} = A + \frac{B}{\mu} + C\mu \tag{6}$$

where u is the linear velocity and A−C are constants. The A term represents the multipath effect, the B term is linked to axial diffusion, and the C term corresponds to mass transfer.[23] HETP was calculated as previously described in the literature, considering the peak of CepC obtained during the elution of the column.[24]

2.4. CepC Recovery Calculation. Recoveries of CepC in the fixed-bed column assays were calculated according to the equation

$$R\ (\%) = \frac{\sum_{j=i}^{N} C_j V_j}{C_0 V_0} \tag{7}$$

where C_0 and V_0 are the concentration of the solution feed and the volume applied into the column, respectively; C_i and V_i are the concentration and volume, respectively, of the fraction collected at the column outlet, and i and N are the first and last fractions, respectively, collected in each step of column operation. The calculations were carried out for each step of the column operation (feed, washing and elution). The total recovery consisted of the sum of the recoveries of all steps.

2.5. Solid-Phase Extraction. Solid-phase extraction was carried out with the fractions containing CepC collected in the ion-exchange column before their injection into the mass spectrometer. This process was carried out to remove the salts from the fractions, which could interfere in the ionization of the molecules in the liquid chromatography−mass spectrometry (LC−MS) system. A C18 SPE cartridge (10 g) (Waters, Dublin, Ireland) was used in the solid-phase extraction. Before sample application, the cartridge was treated by sequentially passing 50 mL of methanol and 30 mL of deionized water through it. After that, the freeze-dried fractions were resuspended in 10 mL of citrate/phosphate buffer (pH 2.6) and applied to the cartridge. The cartridge was then washed with 30 mL of water to remove unbound molecules and eluted with 50 mL of 50% methanol. Fractions of 4 mL were collected for CepC concentration determinations and mass spectrometry analyses. A low buffer pH was used to suppress the ionization of CepC's carboxylic group.

2.6. Analytical Methods. *2.6.1. CepC and Contaminant Determinations.* The concentration of CepC was determined by high-performance liquid chromatography (HPLC) according to the methodology described by Baptista-Neto et al.[25] The analysis was carried out using a 4-μm Synergi MAX RP 12 column (Phenomenex, Torrance, CA) at 28 °C and 0.01 M acetic acid as the mobile phase at a flow rate of 2 mL/min. Peaks were detected at 254 nm. The HPLC system (Waters, Milford, MA) used for the analyses consisted of two pumps, an automatic injector, a temperature controller, and a photodiode array (PDA) detector.

Contaminants were determined by UV absorbance at wavelengths between 310 and 400 nm. Data were collected on an Ultrospect 2100 spectrophotometer (GE Healthcare Europe, Munich, Germany) and stored using Swift II data acquisition software. This methodology detects in the sample all of the compounds that absorb light at these wavelengths and does not detect CepC, which does not absorb at wavelengths above 310 nm.[25] The contaminants were quantified in terms of the area under the curve of wavelength versus absorbance.

2.6.2. Antimicrobial Activity. The antimicrobial activities of the fractions collected during the ion-exchange experiments were estimated according to the method described by Pérez-Redondo et al.,[26] which is based on growth inhibition zones formed in agar previously seeded with a test microorganism. The solution to be tested was poured into wells made in the agar plate, and after incubation, the inhibition zones were measured. *Escherichia coli* (ESS), which is a bacterium sensitive to CepC, was the microorganism used in these assays.

2.6.3. LC−MS Analyses. Liquid chromatography−mass spectrometry (LC−MS) analyses were carried out using Thermo Finnigan LXQ equipment (Waltham, MA) composed of a pump, an autosampler, and a PDA detector coupled to a mass spectrometer. A Hypersil Gold C18 column (100 × 4.6 mm) (Thermo Electron Corporation, Madison, WI) with a particle size of 5 μm was used in the analyses. The mobile phase consisted of methanol/water (70:30 v/v) with 0.1% acetic acid

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and was used at a flow rate of 0.4 mL/min. The ion-trap mass spectrometer was equipped with an electrospray ion (ESI) source, and ionization was performed in positive mode. The capillary temperature was set to 300 °C, and the voltages of the source and capillary were 5 kV and 3 V, respectively. The sheath, auxiliary, and sweep gas flows used were 70, 30, and 30 units, respectively.

2.7. Chemicals. All chemicals used in this study were of analytical grade, with the exception of acetic acid (Mallinckrodt, Hazelwood, MO) and methanol (J.T. Baker, Ecatepec, Mexico), which were of HPLC grade. NaCl was purchased from QHEMIS (Jundiaí, Brazil). Phosphate/citrate buffer was prepared by dissolving appropriate masses of anhydrous citric acid (QHEMIS, Jundiaí, Brazil) and sodium phosphate dibasic heptahydrate (Synth, São Paulo, Brazil) in the fractions collected by ion exchange.

3. RESULTS AND DISCUSSION

3.1. Ion-Exchange Column Processes on QXL Resin. The experimental breakthrough curves exhibited similar profiles (Figure 1). An unusual phenomenon was observed: During a certain period of time, the CepC concentration in the column outlet reached higher values than that in the feed ($C > C_0$). This behavior can be explained by a sequential ion-exchange mechanism that occurs because of the competition of different ionic species for the adsorption sites.[27] As a consequence, when no more sites were available for adsorption, contaminants in the bulk were able to displace the already adsorbed CepC, which was released into the liquid phase. This might explain why, during a certain period of time, there was more CepC in the column effluent than in the feed, and it also explains the reduction in the concentrations of the contaminants after column saturation. Competitive adsorption has also been observed during the ion exchange of metals in zeolites, the separation of α-lactalbumin from bovine serum albumin,[28] the removal of acetic acid and butanoic acid from industrial condensate of a beet distillery,[29] and the removal of nitrate from groundwater.[30]

The efficiencies, productivities, and other process parameters calculated from the breakthrough curves are summarized in Table 1. As expected, the results showed that the process operated at the highest flow rate reached the breakthrough time faster. Significant differences were not observed for the efficiencies (ϕ_1 and ϕ_2), indicating that the flow rate did not interfere with CepC adsorption. The high values obtained for the product recovery efficiency (ϕ_2) suggest a good affinity between CepC and the active sites of the resin. Additionally, the values of bed utilization efficiency (ϕ_1) were also high (92−94%), showing that the resin bed was used to nearly its full capacity. The best productivity value was obtained in the assay using the highest flow rate.

The desorption profiles for each assay are shown in Figure 2, and Table 2 reports the HETP values obtained. A crescent tendency in the HETP values with increasing linear velocity was observed, which is the behavior predicted by the van Deemter equation C term. This suggests that, for the range of flow rates evaluated, the mass transfer between the liquid bulk and the resin was the main mechanism affecting the efficiency of the column. Considering this analysis, the lowest flow rate was more appropriate for the elution step. CepC was recovered in 0.1 L of 0.1% NaCl solution during assay CR1, whereas it was recovered in volumes that were 20% and 40% larger during assays CR2 and CR3, respectively.

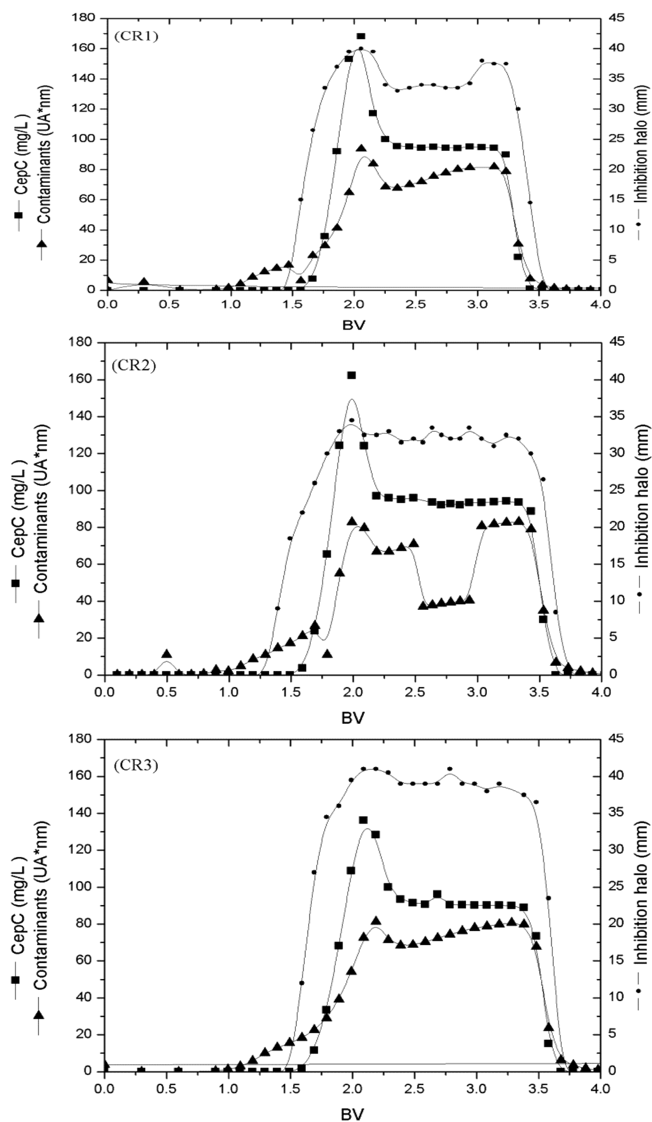

Figure 1. Profiles of cephamycin C, contaminants, and antimicrobial activity during feed application and part of the washing step in fixed-bed columns with Q Sepharose XL resin. CR1, 2.5 mL/min (5.3 m/s); CR2, 5.0 mL/min (10.6 m/s); CR3, 7.5 mL/min (15.9 m/s). Values are given in flow rates (corresponding linear velocity).

Table 1. Parameters Calculated from Breakthrough Curves in Fixed-Bed Columns Packed with Q Sepharose XL Resin

parameter[a]	CR1	CR2	CR3
t_b (min)	13.60	6.40	4.53
q_t (mg/mL)	0.17	0.16	0.19
ϕ_1	0.94	0.92	0.92
ϕ_2	0.99	0.99	0.99
P (mg/min)	0.36	0.71	1.16

[a] t_b, break-point time; q_t, amount of CepC adsorbed per bed volume until column saturation; ϕ_1, efficiency of bed utilization; ϕ_2, product recovery efficiency; P, productivity.

The proposed elution method was able to separate CepC from other antibiotics present in the broth, as two separate fractions with antibacterial activity against *E. coli* were detected (Figure 2). The first peak was obtained by elution with 0.1% NaCl solution, whereas the second peak was detected during elution with 0.5% NaCl solution. HPLC analyses revealed that

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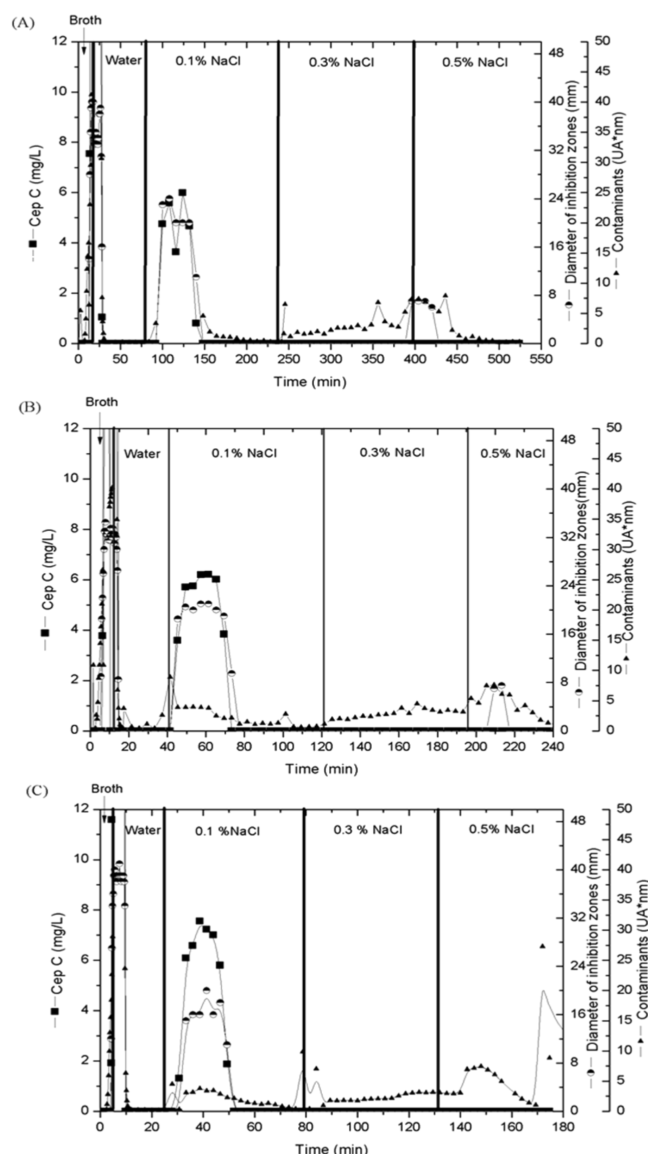

Figure 2. Adsorption and elution profiles of cephamycin C, compounds with antibacterial activity and contaminants in fixed-bed columns with Q Sepharose XL resin at three flow rates (corresponding linear velocities): (A) 2.5 mL/min (5.3 m/s), (B) 5.0 mL/min (10.6 m/s), (C) 7.5 mL/min (15.9 m/s).

Table 2. HETP Values Obtained during the Desorption Step of the Ion-Exchange Process for Different Linear Velocities

	u ($\times 10^{-4}$ m s^{-1})	HETP ($\times 10^{-3}$ m)
CR1	5.3	1.8
CR2	10.6	2.8
CR3	15.9	3.5

Table 3. Recovery of Cephamycin C in Fixed-Bed Column Processes Using QXL Resin

		recovery (%)			
assay	flow rate (mL/min)	total	feed	washing	elution (0.1% NaCl)
CR1	2.5	85.8	43.4	30.8	11.5
CR2	5.0	92.2	38.9	36.9	16.4
CR3	7.5	88.3	32.3	37.9	18.2

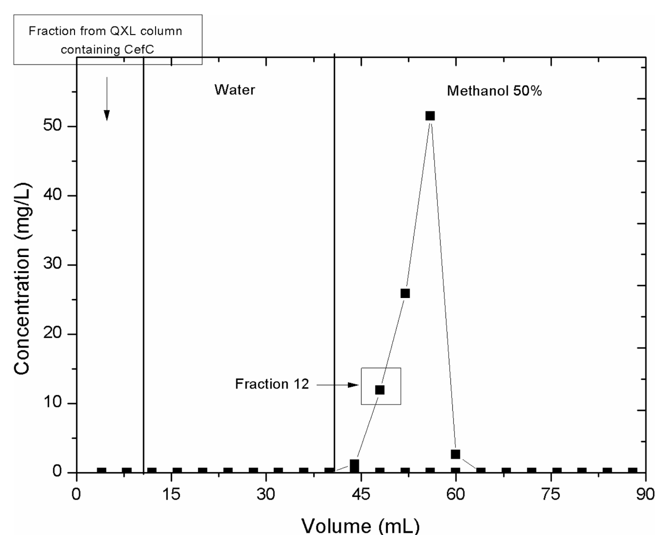

Figure 3. Cephamycin C profile during fraction application, washing, and elution during solid-phase extraction on a C18 cartridge. The fraction consisted of the purified peak of CepC obtained after ion exchange. Washing was carried out using deionized water, and methanol (50%) was used in elution.

CepC was present only in the first peak. The compounds present in the second peak were not identified; however, they might be intermediates of the biosynthetic pathway of CepC that can also be produced in small quantities and secreted to the culture medium and also have antibacterial activity.[31]

The contaminant profiles showed that some of these compounds were released together with CepC, with the rest being released during the elutions with 0.3% and 0.5% NaCl solutions. Therefore, the proposed methodology was able to separate CepC from some contaminants present in the feed, but the fraction obtained was not yet in pure form.

The CepC recoveries after the ion-exchange processes are reported in Table 3. The total recovery of CepC was high for all of the processes evaluated, ranging near 90%. The increase in the operating flow rate showed a tendency to increase the CepC recovery. The highest amount of CepC was recovered during the feed and washing steps. This probably occurred because the column was fed beyond bed saturation, as, at this point of the study, the CepC breakthrough point was not known. After obtaining the breakthrough curves, it was possible to determine the time at which the loading of the column should have stopped, called the breakthrough time (t_b). In practice, the column should be loaded until a small amount of CepC is detected in the effluent of the column ($C/C_0 < 0.1$). In this way, loss of CepC would be smaller in the initial steps, and most of the antibiotic would be recovered during elution.

As the efficiencies were not affected by the flow rate, for the feed step, a flow rate of 7.5 mL/min was selected among the tested operating conditions as being the most suitable to be used in this process, because it promoted the best productivity value. For the desorption step, the flow rate of 2.5 mL/min was the best, as it resulted in the lowest value of HETP obtained.

Baptista-Neto et al.[5] proposed a process for CepC purification using the same resin (Q Sepharose XL) as used in the present study. However, the fixed-bed experiments that they proposed using QXL resin were not able to separate different peaks, and only one large fraction containing CepC in a mixture with other compounds was obtained during the elution step.

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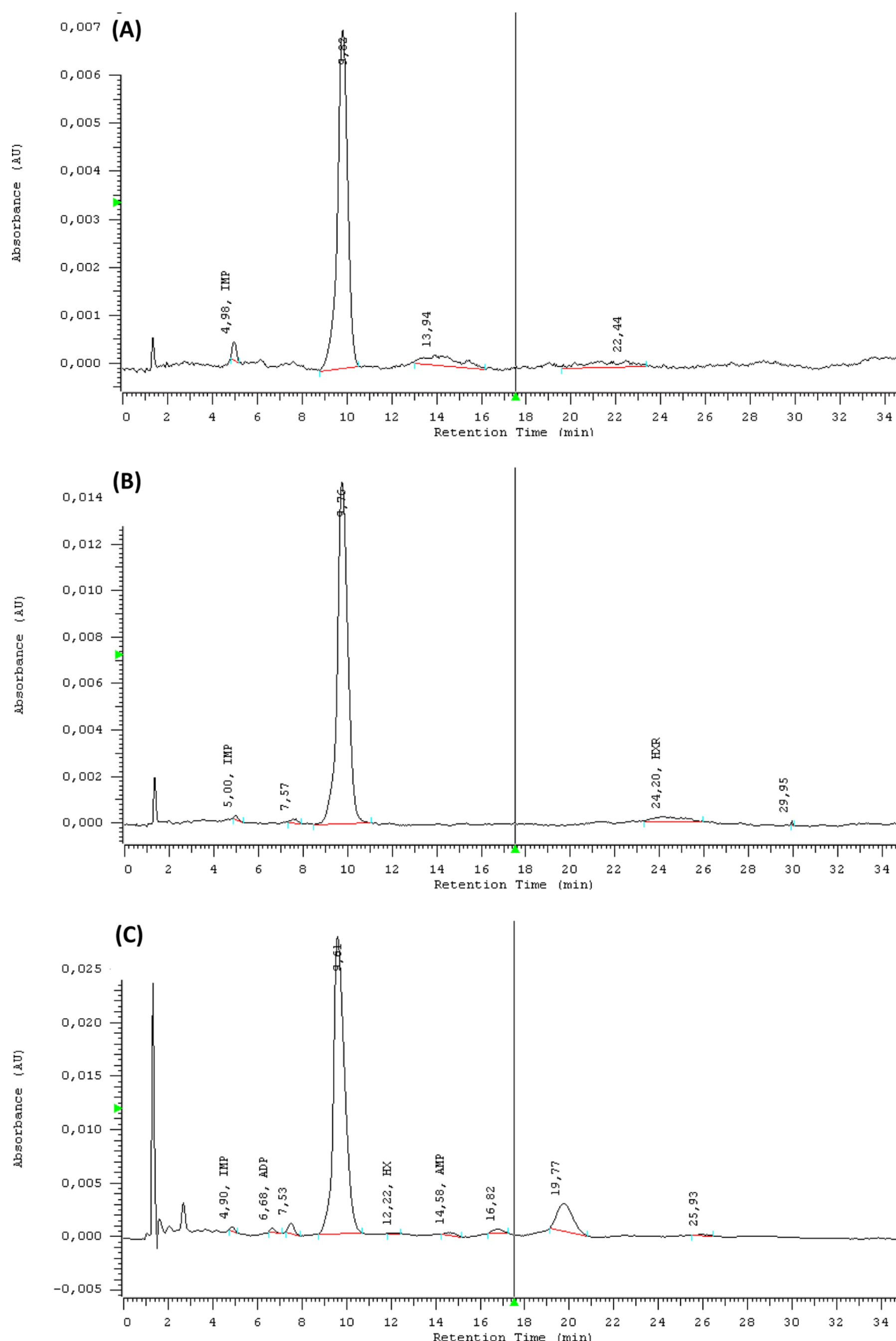

Figure 4. UV chromatogram at 254 nm for fractions (F) obtained during solid-phase extraction using a C18 cartridge: (A) F12, (B) F13, and (C) F14.

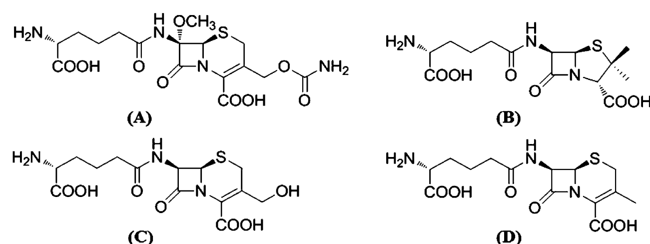

Figure 5. Molecular structures of (A) cephamycin C, (B) penicillin N, (C) deacetylcephalosporin C, and (D) deacetoxycephalosporin C.

3.2. Qualitative Evaluation of CepC Purification after Ion Exchange on QXL Resin Followed by Solid-Phase Extraction on a C18 Cartridge by Mass Spectrometry.

As discussed above, a partially purified peak of CepC was obtained after the ion-exchange column process. It was not possible to carry out a quantitative analysis of the contaminants because chemical standards for these compounds are not commercially available and there is no alternative methodology for determining them. To evaluate the purification attained, samples of this peak and of the clarified broth were analyzed by mass spectrometry.

Prior to their injection into the mass spectrometer, the fractions collected in the ion-exchange assays were subjected to a desalination process using solid-phase extraction. Figure 3 shows the CepC profiles during the feed application, washing, and elution steps. Four fractions were collected during elution

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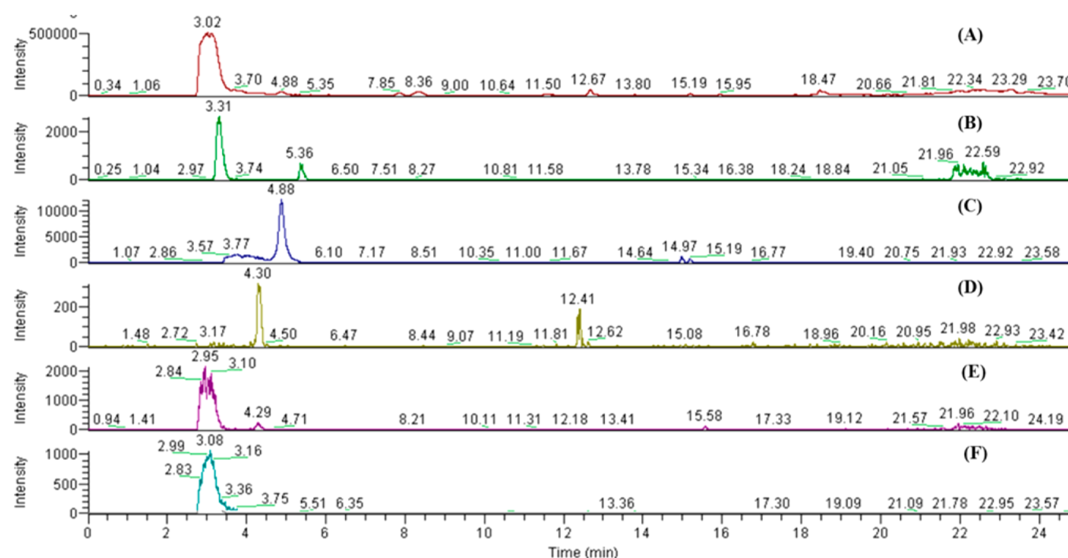

Figure 6. (A) Total-ion chromatogram (TIC) and (B−F) base-peak chromatograms of the clarified broth used in the ion-exchange column process. Base-peak chromatograms were obtained by selecting the *m*/*z* ratio for each monitored compound, as follows: (B) cephamycin C (*m*/*z* 447), (C) penicillin N (*m*/*z* 360), (D) deacetylcephalosporin C (*m*/*z* 374), (E) deacetoxycephalosporin C (*m*/*z* 358), (F) lysine (*m*/*z* 147).

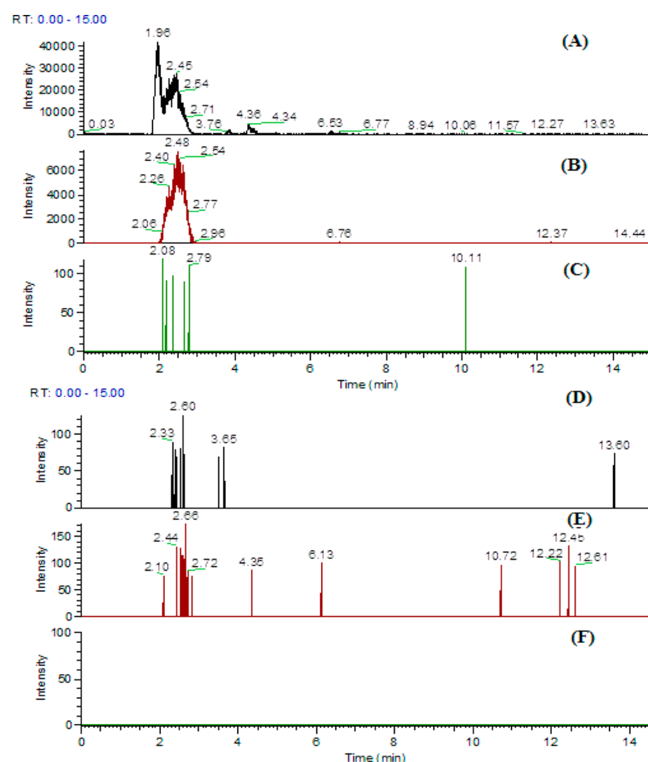

Figure 7. (A) Total-ion chromatogram (TIC) and (B−F) base-peak chromatograms of F12 obtained during solid-phase extraction. Base-peak chromatograms were obtained by selecting the *m*/*z* ratio for each monitored compound, as follows: (B) cephamycin C (*m*/*z* 447), (C) penicillin N (*m*/*z* 360), (D) deacetylcephalosporin C (*m*/*z* 374), (E) deacetoxycephalosporin C (*m*/*z* 358), (F) lysine (*m*/*z* 147).

with 50% methanol. The chromatograms obtained at 254 nm suggested that fractions 12 (F12) and 13 (F13) had high degrees of purity (Figure 4A,B), as they showed a major peak corresponding to CepC and other very small peaks corresponding to contaminants. When compared to the other fractions, fraction 14 (F14) (Figure 4C) was more concentrated in CepC, but it also contained three or four other minor peaks.

F12 was then collected and injected into the LC−MS instrument, as well as a sample of the clarified broth. The ionization was carried out in positive mode; therefore, the *m*/*z* ratio detected for each molecule corresponded to its molecular mass plus 1. The total-ion chromatogram of each sample was obtained. Afterward, the base-peak chromatograms were extracted by selecting specified ranges of *m*/*z* ratios. The *m*/*z* ranges chosen corresponded to the molecular mass of the protonated monitored molecule plus or minus 0.5. This narrow range was used to avoid the detection of different compounds in the base-peak chromatograms.

The signals (*m*/*z* ratios) monitored in these analyses were the signals generated by ions corresponding to a molecule of CepC and to molecules of some compounds that could be contaminants. The molecules investigated as possible contaminants consisted of some intermediates in the CepC biosynthetic pathway, namely, deacetoxycephalosporin C (*m*/*z* 358), deacetylcephalosporin C (*m*/*z* 374), and penicillin N (*m*/*z* 360). Previous studies showed that the broth also contained small amounts of these compounds.[31] As these compounds have structures that are very similar to that of CepC (Figure 5), they could have similar affinities for the ion-exchange resin and the adsorbent used, in which case they could not have been separated. The other compound monitored in this analysis was the amino acid lysine (*m*/*z* 147), which is added to the culture medium in large amounts.

Figures 6 and 7 show the total-ion and base-peak chromatograms obtained for the clarified broth and for F12, respectively. The chromatograms obtained revealed that some of the selected compounds were present in both the clarified broth and F12. Nevertheless, when observing the base-peak chromatograms, it is noteworthy that the ratios of the monitored contaminants to CepC were diminished in F12 compared to the clarified broth and that lysine was not detected in F12.

The mass spectrometry methodology used to verify the separation of CepC from the monitored contaminants did not permit a separate evaluation of the ion-exchange process, as the desalination step is necessary prior to injection into the LC−MS system. Possibly, adsorption on the C18 adsorbent also

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promoted a purification of the fractions collected in the ion-exchange column. Therefore, the results demonstrated that the ion-exchange process followed by a chromatographic process on a C18 adsorbent is a suitable procedure for purifying CepC from fermentation broth.

4. CONCLUSIONS

A fixed-bed column process using the anionic resin Q Sepharose XL was able to separate CepC from fermentation broth. The use of flow rates varying between 2.5 and 7.5 mL/min did not influence the bed utilization or the resin uptake capacity. The highest flow rate (7.5 mL/min) provided the best productivity values during the feed step of the process, whereas the lowest flow rate (2.5 mL/min) provided the best column efficiency during desorption. A stepwise gradient for column elution using NaCl solution was able to separate a peak containing CepC from another peak containing other antibiotics. However, other compounds were also eluted together with CepC. A subsequent chromatographic process on a C18 adsorbent decreased the concentration of contaminants in the sample, improving the CepC purification results. This process was able to separate CepC from a compound with an m/z ratio corresponding to that of lysine. Additionally, compounds with m/z ratios corresponding to some contaminants, namely, deacetylcephalosporin C, deacetoxycephalosporin C, and penicillin N, also had their concentrations reduced after this process. It was then concluded that the ion-exchange process followed by adsorption on a C18 adsorbent is an efficient method for purifying CepC from fermentation broth. This result is of great interest taking into account the current necessity of establishing a process for CepC purification from fermentation broth.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: S.I.Mussatto@tudelft.nl, solangemussatto@hotmail.com. Tel.: +31 0 15 278 8789. Fax: +31 0 15 278 2355.

Notes

The authors declare no competing financial interest.

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