

## **Materials and methods**

### *Synthesis and purification of PCS*

PC and IS were purchased commercially from Sigma-Aldrich (St. Louis, MO, USA). The synthesis of PCS was performed as described by Maciel et al. 2016 [32], with few modifications, basically by reacting ice-cold solution of PC (10 g, 0.084 M) with chloroform and pyridine (2:1). The sulfation was carried out by the drop-by-drop addition of chlorosulfonic acid with constant stirring (10 mL, 0.129 M). The mixture was made alkaline with strong aqueous potassium hydroxide (20 M) resulting in an ester potassium salt as shown in Figure 1 [33]. The solid portion was filtered through a *filter* paper in a *Buchner* funnel by suction and washed thoroughly with ether. The ester salt was extracted from the residue with hot isopropyl alcohol (95%). The compound was purified by column chromatography with silica gel 60-230 mesh (1:20, Merk, Darmstadt, Germany) by increasing the eluent polarity, beginning with chloroform (100%) and then, with increments of methanol (fractions F1-8) to afford PCS (2.56 g, 92%) as a white solid. The purification of PCS was accomplished by high-performance liquid chromatography (HPLC) whereas the chemical characterization was performed by ultra-high-performance liquid chromatography with high-resolution mass spectrometry (UHPLC-HRMS) and nuclear magnetic resonance (NMR). Stock solutions were prepared at concentrations of 1000 mg/L and 5000 mg/L, diluted in Krebs-Ringer phosphate (KRP) buffer, and stored at -20°C. The final PCS concentrations were (0.08, 1.75, and 2.6 mg/L) obtained by the dilution of the stock solution using KRP buffer.

### *Chemical characterization of PCS*

In order to monitor the purification steps, the fractions were analyzed by HPLC (Agilent 1100 series) with binary pump Agilent 1100 and a fluorescence spectrophotometer. The reversed phase chromatography was developed with Microsorb MV 100 C18 (250 x 4.6 mm), particle size 5  $\mu\text{m}$  (CicaMerk, Tokyo, Japan), in 100% methanol (A) and 50 mM ammonium formate (B) with the following gradient of B concentration: 65-25% (0-15 min) and 25-65% (15-20 min). The sample consisted of cell lysates and the chromatography was performed at a flow rate of 1.0 mL/min; 10  $\mu\text{L}$  of the sample was injected and the fluorescence was detected at an excitation and emission wavelengths of 214 and 306 nm, respectively. PCS was confirmed in the fractions by LC-MS, performed with an ultra-performance liquid chromatography (UPLC<sup>TM</sup>, Acquity, Waters), with a binary pump, sample delivery column, oven, and photodiode array detector (PDA). The column was a HSS-T3 C18 (Waters) with 100 x 2.1 mm and particle size of 1.7  $\mu\text{m}$  and the solvents were type 1 water (A) and methanol (B), both containing 0.1 % formic acid. The chromatography was performed at 60°C at a flow rate of 400  $\mu\text{L}/\text{min}$  by increasing B concentrations from 5-60% (5 min), 60-100% (6 min), held for 7 min, and then returning to the initial condition in 7.1 min with 3 min for system re-equilibration. The sample (1 mg) was dissolved in 1 mL of MeOH-H<sub>2</sub>O (1:1, v/v) and 10  $\mu\text{L}$  was injected. The detection was performed by PDA (200-400 nm) and high-resolution mass spectrometry (HR-MS), using an electrospray apparatus LTQ OrbiTrap XL (Thermo-Scientific), with atmospheric pressure ionization detected in the negative ionization mode ( $m/z$  80-500). Nitrogen was used for sample desolvation with sheath gas at a flow rate of 60 arbitrary units (a.u.) and auxiliary gas of 20 a.u., at a source temperature of 350 °C. The ionization energies were 3.5 kV on spray voltage, -

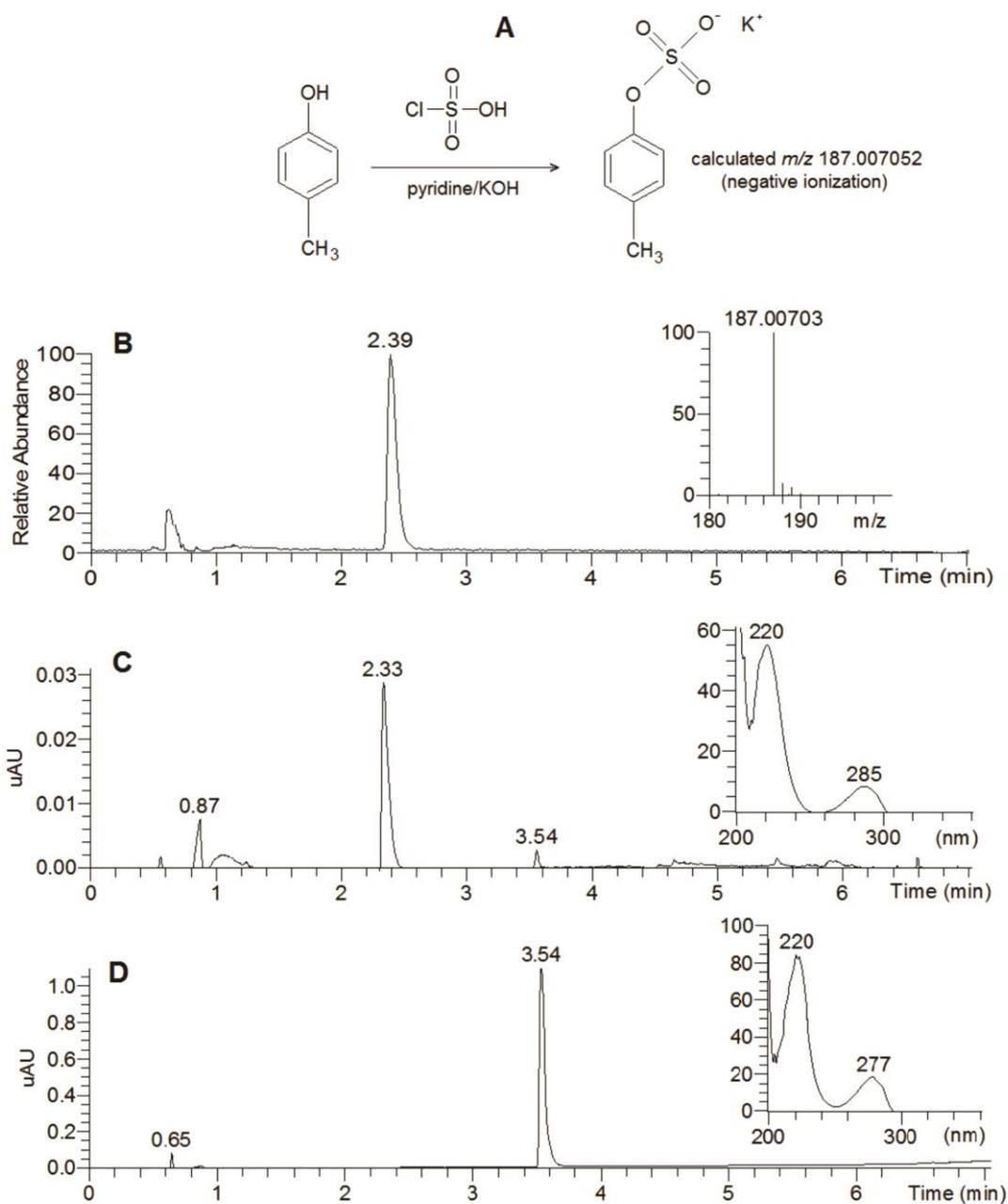
40 V on capillary and -120 V on tube lens. For mass accuracy, the external calibration was performed ( $m/z$  100-2000) and resolution was set at 7,500 FWHM. The PCS-containing fraction (S) was also analyzed by NMR and recorded by a BrukerAvance III 600 MHz spectrometer (Bruker; Karlsruhe, Germany) with an inverse probe of 5 mm. Chemical shifts ( $\delta$ ) are given in ppm, with deuterated water ( $D_2O$ ) as solvent and 3-(trimethylsilyl) propionic 2,2,3,3-d<sub>4</sub> acid as an internal standard ( $^1H$ ,  $^{13}C$  = 0 ppm).

## Results

### *Characterization of PCS*

HPLC showed that fractions 5 and 6 contained PCS; thus, these fractions were used for LC-UV-MS analysis. By MS detection, a main peak (1) has appeared at 2.39 min, with a negative ion at  $m/z$  187.00703, consistent with PCS that had a calculated  $m/z$  of 187.007052 [ $C_7H_7O_4S$ ]<sup>-</sup> (Fig. 1A,B). The precursor (PC) was not detected by negative ESI-MS, however by UV detection a low abundant peak (2) with a retention time of 3.54 min has appeared together with the main peak (92%) at 2.33 min (Fig. 1C). The UV-spectra of both were similar with  $\lambda_{max}$  at 220-285 nm (peak 1) and 220-277 nm (peak 2). Peak 2 was confirmed as PC by comparison with the standard (Fig. 1D). The NMR spectroscopic data of PCS is as follows:  $^1H$  NMR ( $D_2O$ , 600 MHz):  $\delta$  2.31 (s, 3H, H<sub>8</sub>), 7.18 (d, 2H,  $J$  8.80, H<sub>2-6</sub>), 7.24 (d, 2H,  $J$  8.40, H<sub>3-5</sub>);  $^{13}C$  NMR ( $D_2O$ , 600 MHz):  $\delta$  20.0 (C<sub>8</sub>), 121.41 (C<sub>2-6</sub>), 130.24 (C<sub>3-5</sub>), 136.5 (C<sub>4</sub>), 148.99 (C<sub>1</sub>).

**Figure 1. Characterization of PCS**



**Figure 1. (A) Schematic representation of the synthesis of *p*-cresyl sulfate (PCS).** The reaction was accompanied by LC-PDA-MS analysis; the main peak (2.39 min) observed from F6 fraction (B) gave the ion at  $m/z$  187.00703 (calculated value from anionic PCS  $m/z$  187.007052). Observation with PDA (C) gave a low abundant peak (3.54 min), similar to the authentic standard of PC (D).