

1                   **Methacrylic functionalized hybrid carbon nanomaterial for the selective adsorption and**  
2                   **detection of progesterone in wastewater**

3                   **Xia Cui <sup>a,b</sup>, Hua Shu <sup>a,b</sup>, Lu Wang <sup>a,b</sup>, Guoning Chen <sup>a,b</sup>, Jili Han <sup>a,b</sup>, Qianqian Hu <sup>a,b</sup>,**  
4                   **Kamran Bashir <sup>a,b</sup>, Zhimin Luo <sup>a,b</sup>, Chun Chang <sup>a,b</sup>, Jia Zhang <sup>c</sup>, Qiang Fu <sup>a,b,c,\*</sup>.**

5                   <sup>a</sup> Department of Pharmaceutical Analysis, School of Pharmacy, Xi'an Jiaotong University, Xi'an  
6                   710061, China

7                   <sup>b</sup> Institute of Drug Safety and Monitoring, Academy of Pharmaceutical Science and Technology, Xi'an  
8                   Jiaotong University, Xi'an 710061, China

9                   <sup>c</sup> Shaanxi Hanjiang Pharmaceutical Group Co., Ltd, Hanzhong 723000, China

10                  \* Corresponding author at: Department of Pharmaceutical Analysis, School of Pharmacy, Xi'an  
11                  Jiaotong University, Xi'an 710061, China, and Institute of Drug Safety and Monitoring, Academy of  
12                  Pharmaceutical Science and Technology, Xi'an Jiaotong University, Xi'an 710061, China

13                  E-mail address: fuqiang@mail.xjtu.edu.cn (Q. Fu)

14                  Telephone and fax number: 029-82655382.

15

16 **Abstract**

17 Progesterone, an endocrine-disrupting chemical, has been frequently detected in wastewater for  
18 decades, posing a serious threat to ecological and human health. However, it is still a challenge to achieve  
19 the effective detection of progesterone in complex matrices water samples. In this study, a novel  
20 adsorbent CNT@CS/P(MAA) was prepared by grafting methacrylic polymers on the surface of modified  
21 carbon nanomaterials. Compared with other reported materials, the hybrid carbon nanomaterial could  
22 selectively identify the progesterone in the complex industrial pharmaceutical wastewater, and its  
23 adsorption performance is almost independent of pH and environmental temperature. In addition, this  
24 nanomaterial could be reused with a good recovery rate. The prepared nanomaterials were characterized  
25 by transmission electron microscopy, Fourier transform infrared spectroscopy, X-ray diffraction,  
26 nitrogen adsorption and desorption experiments and thermo gravimetric analysis. The results confirmed  
27 that the methacrylic polymers and chitosan layer were successfully grafted on the surface of carbon  
28 nanotubes. Adsorption isotherms, adsorption kinetics, and selectivity tests showed that  
29 CNT@CS/P(MAA) had a high adsorption capacity ( $44.45 \text{ mg}\cdot\text{g}^{-1}$ ), a fast adsorption rate and a satisfied  
30 selectivity for progesterone. Then, CNT@CS/P(MAA) was used as solid phase extraction sorbent and  
31 combined with HPLC to enrich progesterone from the wastewater samples. Under the optimum  
32 conditions, a good linearity was obtained with the correlation coefficient was 0.9998, and the limit of  
33 detection was  $0.003 \text{ ng}\cdot\text{mL}^{-1}$ . Therefore, this method could be used for the selective and effective  
34 detection of progesterone in the industrial wastewater with complex substrates, and provided a new  
35 method for the detection of progesterone in other environmental waters.

36 **Keywords**

37 Progesterone; Methacrylic; Hybrid carbon nanomaterial; Solid phase extraction; Endocrine-disrupting  
38 chemical; Wastewater.

39

40 **Highlights**

- 41 1. A methacrylic functionalized hybrid carbon nanomaterial was synthesized.
- 42 2. The prepared nanocomposites could selectively recognize and enrich P4 in water.
- 43 3. The adsorption capacity of this material for P4 is  $44.45 \text{ mg}\cdot\text{g}^{-1}$ .
- 44 4. The adsorption performance of P4 is hardly affected by pH and adsorption temperature.
- 45 5. The SPE-HPLC method could be used to detect P4 in real industrial wastewater.
- 46

## 47 **1. Introduction**

48 The presence of endocrine-disrupting chemicals (EDCs) in the environment has attracted extensive  
49 attention from the public for their ability to impair reproductive and immune functions of aquatic  
50 organisms (Du et al. 2018, Zhang et al. 2017). These chemicals include natural and synthetic hormones  
51 as well as their metabolites (Hashmi et al. 2020). Previous researches mostly focused on estrogen and  
52 androgens (Luque-Cordoba et al. 2020, Tian et al. 2020, Zhang et al. 2020b). However, there is growing  
53 awareness that other EDCs in the environment, such as progesterone (P4), may also affect the health of  
54 aquatic organisms and human (Fabbrocini et al. 2019, Hashmi et al. 2018).

55 Progesterone (Fig. 1), a C-21 structural steroid hormone, can help to modulate the menstrual cycle,  
56 making the uterus ready for pregnancy, and affect the breast development and lactation (Graham  
57 & Clarke 1997). It has been widely applied in breast cancer therapy, endocrine regulation, drug  
58 contraception and assisted reproduction (De Lima & Spinelli 2013, Guohua et al. 2017, Kumar et al.  
59 2015). However, it has become a main contaminant in the aquatic environment due to the massive  
60 discharge of industrial waste and urban sewage (Hashmi et al. 2020). In China, the production and  
61 application of P4 is huge, due to the front-end advantage of natural resources of diosgenin plants such  
62 as *Dioscorea zingiberensis* (Guo et al. 2016). At the same time, P4 can be used as a synthetic precursor  
63 of other steroid hormones, which also increases the production of P4. Once a large amount of P4  
64 remaining in industrial production wastewater is discharged into the environment, it will pose a great  
65 threat to animal and human health. (Kumar et al. 2015, Yu et al. 2019). However, it is still a challenge  
66 to effectively detect and enrich the residual P4 in the industrial wastewater due to the complex substrate,  
67 many interfering coexisting substances and wide range of pH values. Therefore, developing an effective  
68 method to monitor P4 in the industrial waste is necessary. Considering the concentration of P4 in the  
69 industrial water, such methods not only suit for the complex media, but also require high sensitivity.

70 To date, several analytical methods such as thin layer chromatography (TLC) (Chamas et al. 2017),  
71 gas chromatography (GC) (Nezhadali et al. 2016), high performance liquid chromatography (HPLC)  
72 (Kollofrath et al. 2020), capillary electrophoresis (Siren & El Fellah 2017), gas chromatography-mass  
73 spectrometry (GC/MS) (Siren & El Fellah 2017), liquid chromatography-mass spectrometry (LC-MS)  
74 (Zong et al. 2018), electrochemical sensor (Akshaya et al. 2019), and enzyme linked immunosorbent  
75 assay (ELISA) tests (Zhang et al. 2020c) have been used to determine the presence of P4 in environmental  
76 samples, especially in the wastewater. HPLC is the most frequently used method because of the low cost,  
77 high performance, relatively good sensitivity and stability. Roya Mirzajani et al. (Mirzajani et al. 2019a)  
78 reported a nanocomposite consisting of graphene oxide, ZIF-8 metal-organic frameworks and  
79 molecularly imprinted polymers, which was used as the sorbent of solid phase microextraction coupled  
80 with HPLC to recognize and separate five sterol and steroid hormones in biological samples. This method  
81 could detect five sterol and steroid hormones simultaneously and had a low detection limit (3-5 ng·L<sup>-1</sup>).  
82 However, it is limited by the pH of the samples, and had some problems such as low adsorption capacity  
83 and potential template leakage. Kasre Razmkhah et al. (Khan et al. 2019) synthesized a magnetic carbon  
84 nanotube-based strontium-titanium (Fe/CNT-SrTiO<sub>3</sub>) adsorbent, which could extract 17β-estradiol,  
85 ethinyl estradiol and progesterone from milk coupled with MSPE-HPLC. This method had good stability  
86 but poor specificity. In order to improve the specificity of analytical methods, biological molecules such

87 as antibodies (Guo et al. 2019), enzymes (Xu et al. 2020) and DNA aptamers (Tao et al. 2020) were used  
88 as recognition tools. Khan Muhammad et al.(Khan et al. 2019) devised an ultra-sensitive  
89 electrochemical-digital sensor chip for monitoring cortisol in real time with anti-cortisol antibody as  
90 recognition elements. Xu Xuan et al. (Xu et al. 2018) fabricated an electrochemical enzymatic  
91 nanoreactor by immobilizing CYP3A4 inside polydopamine modified nanoporous graphene foams,  
92 which was successfully applied to detect three steroid hormones. Those methods had achieved superior  
93 selectivity, but often suffer from unstable, easily deactivated and high price, and cannot be applied to  
94 complex industrial wastewater. Thus, it is urgent to develop a novel recognition material for industrial  
95 wastewater with simple, stable, excellent adsorption performance and high specificity.

96 Nowadays, carbon nanotubes (CNTs) have received much attention due to their outstanding  
97 properties, such as narrow pore distribution, large ( $100$  to  $\geq 500$   $\text{m}^2\cdot\text{g}^{-1}$ ) specific surface area, and  
98 excellent thermal and mechanical resistance (Shu et al. 2020, Xu et al. 2017). However, the use of CNTs  
99 is limited for their easy agglomeration, hydrophobicity and biological toxicity (Jakubus et al. 2016).  
100 Studies have shown that the surface-carrying groups of CNTs can be changed by surface modification to  
101 improve their properties (Chen et al. 2018, Liu et al. 2018). In our previous study, we reported a method  
102 of modifying carbon nano-material with chitosan. The prepared carbon microcoil-chitosan composites  
103 showed good solubility, dispersibility and pH-controllability (Hua et al. 2018).

104 Herein, we intend to fabricate a selective sorbent by grafting the methacrylic acid polymer onto the  
105 surface of carbon nanotubes modified with chitosan. The prepared material was served as the adsorbent  
106 of solid phase extraction column and combined with high-performance liquid chromatography (HPLC)  
107 for the selective enrichment and determination of P4. The hybrid carbon nanomaterial modified with  
108 methacrylic acid polymers (CNT@CS/P(MAA)) was analyzed by transmission electron microscopy  
109 (TEM), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), nitrogen adsorption  
110 and desorption experiments and thermo gravimetric analysis (TGA). The adsorption isotherms and  
111 kinetics were studied to elucidate the adsorption mechanism. Moreover, the pH value, temperature and  
112 other parameters affecting adsorption efficiency and recovery were also investigated. Finally, the  
113 established SPE-HPLC method was applied to detect P4 in the real industrial wastewater samples.

## 114 **2. Experiment**

### 115 **2.1. Reagents and chemicals**

116 Progesterone, estrone (E) and estradio (E2) were purchased from Wuhan Dong Kangyuan  
117 Technology (Hubei, China). Dexamethasone (DXM), bisphenol A (BPA) and diethylstilbestrol (DES)  
118 were provided by Hubei Kang Baotai Fine Chemical (Hubei, China). Methacrylic acid (MAA) was  
119 provided by Tianjin Chemical Reagent Plant (Tianjin, China) and distilled under vacuum to remove  
120 inhibitors before use. Ethylene glycol dimethacrylate (EGDMA) was obtained from Sigma–Aldrich  
121 (New Jersey, USA) and refined by distillation. 2,2'-azobisisobutyronitrile (AIBN) was purchased from  
122 Shanghai No.4 Reagent Factory (Shanghai, China) and recrystallized from methanol before use. Chitosan  
123 ( $\text{MW} = 600000$   $\text{g}\cdot\text{mol}^{-1}$ ) with a 90% degree of deacetylation was obtained from Sinopharm Chemical  
124 Reagent Co., Ltd (Shanghai, China). Glutaraldehyde (25% aqueous solution) was purchased from Tianjin  
125 Fuchen Chemical Reagents Factory (Tianjin, China). HPLC-grade methanol was purchased from Tianjin  
126 Kemiou Chemical Reagent Co. (Tianjin, China). Ultrapure water was prepared using a Molement 1805b

127 purification system (Shanghai, China). And all other reagents were of analytical grade. Progesterone  
128 standard solutions were diluted with 1.0 mg·mL<sup>-1</sup> progesterone stock solution and prepared temporarily  
129 before use. The real industrial wastewater samples were collected from a local pharmaceutical enterprise  
130 in Hanzhong, Shannxi.

## 131 **2.2. HPLC conditions**

132 A Shimadzu high performance liquid chromatograph system (LC 2010A HT, Kyoto, Japan) with an  
133 ultraviolet detector was employed for the determination of target. The analytical column (4.6×250 mm)  
134 was a 5 μm C18 column. The 90% methanol solution was used as the mobile phase with a flow rate of 1  
135 mL min<sup>-1</sup>. The detection wavelength was 254 nm and the column temperature was kept at 30 °C.

## 136 **2.3. Preparation of CNT@CS/P(MAA)**

137 CNT@CS complex was synthesized according to our previous report (Liu et al. 2017) with slight  
138 modification, which was described in supplementary material. The fabrication of CNT@CS/P(MAA)  
139 was shown below: 500 mg of CNT@CS was mixed with 100 mL of methanol. Subsequently, the MAA,  
140 EGDMA, and initiator AIBN were added in sequence. After 5 minutes of ultrasound, the reaction was  
141 performed in a shaker at 50 °C for 6 hours. The obtained products were rinsed several times with purified  
142 water and methanol to remove the unreacted substances and impurities, and dried at 50 °C overnight. The  
143 resulting compounds were the CNT@CS/P(MAA).

## 144 **2.4. Characterization of CNT@CS /P(MAA)**

145 TEM images were recorded on a JEM2100 transmission electron microscopy (TEM, JEOL Co.,  
146 Japan). FT-IR spectra were performed on a Nicolet iS10 spectrometer (Thermo Fisher Scientific,  
147 America) in the range of 400 to 4000 cm<sup>-1</sup>. The crystalline structures of the obtained materials were  
148 characterized by XRD (Shimadzu, Japan). The surface area of the polymers was measured by nitrogen  
149 adsorption and desorption experiments using an Autochem 2920 physical chemistry analyzer  
150 (Quantachrome, USA). Brunauer–Emmett–Teller (BET) theory and the Barrett–Joyner–Halenda (BJH)  
151 theory were used to calculate the specific surface area and the average pore diameter. TGA was operated  
152 on an SDT Q600 thermogravimetric analyzer (New Castle, USA) with a ramp of 10 °C·min<sup>-1</sup> from room  
153 temperature to 800 °C.

## 154 **2.5. Adsorption test**

### 155 **2.5.1 Optimization of adsorption conditions**

156 In order to explore the optimum conditions of adsorption experiments, the water contents, pH and  
157 adsorption temperature were investigated.

158 The water contents were optimized by following steps: P4 stock solution (1 mg·mL<sup>-1</sup>) was prepared  
159 with methanol and diluted to 50 μg·mL<sup>-1</sup> with various methanol-water solutions. The water content of  
160 the working solutions was ranged from 10% to 80% due to the solubility of progesterone. Subsequently,  
161 2 mL of P4 working solution was mixed with 5 mg of adsorbent and oscillated 90 minutes at 25 °C. After  
162 filtration, the concentration of P4 in the supernatant was detected by HPLC. And the adsorption capacity  
163  $Q$  (mg·g<sup>-1</sup>) was calculated according to the following formula:

$$164 \quad Q = (C_0 - C_f) \times V / m \quad (1)$$

165 where  $Q$  ( $\text{mg}\cdot\text{g}^{-1}$ ) was the adsorption capacity of the sorbent for P4;  $C_0$  ( $\text{mg}\cdot\text{mL}^{-1}$ ) and  $C_f$  ( $\text{mg}\cdot\text{mL}^{-1}$ ) were  
166 the initial and final concentration of P4 in the working solution, respectively.  $V$  (mL) was the volume of  
167 the working solution, and  $m$  (g) was the weight of the sorbent.

168 The pH and adsorption temperature were optimized by the same method as above, except that the  
169 pH value of the aqueous phase (adjusted to 3.0, 5.0, 7.0, 9.0, 11.0 by adding HCl or NaOH) and the  
170 adsorption temperature (25 °C, 35 °C, 45 °C) were changed.

#### 171 2.5.2. Adsorption isotherms

172 Under the optimum conditions, isothermal adsorption experiments were investigated to explore the  
173 adsorption property and principle of the adsorbent to the analyte. The adsorbent was mixed with various  
174 P4 standard solutions of different concentrations (5, 15, 25, 50, 75, 100, 125, 150 and 175  $\mu\text{g}\cdot\text{mL}^{-1}$ ).  
175 After reacting 120 minutes at 25 °C, 35 °C and 45 °C, the supernatants were filtered and determined by  
176 HPLC. The adsorption capacity of the sorbent for P4 at different concentrations was calculated by Eq.  
177 (2),

$$178 \quad Q_e = (C_0 - C_e) \times V/m \quad (2)$$

179 where  $Q_e$  ( $\text{mg}\cdot\text{g}^{-1}$ ) was the adsorption capacity of the sorbent for P4 at different concentrations;  $C_0$   
180 ( $\text{mg}\cdot\text{mL}^{-1}$ ) represented the initial concentration of P4 in the working solution;  $C_e$  ( $\text{mg}\cdot\text{mL}^{-1}$ ) was the final  
181 concentration of P4 in different solution;  $V$  (mL) was the volume of the working solution, and  $m$  (g) was  
182 the weight of the sorbent.

#### 183 2.5.3. Adsorption kinetics

184 To study the adsorption process and determine the equilibrium time, an adsorption kinetics  
185 experiment was carried out. The specific operation was consistent with the description in the section of  
186 2.5.2, except that the adsorption time was changed to 0.17, 1, 5, 10, 30, 60, 120 and 150 minutes. The  
187 adsorption capacity of the sorbent for P4 at different time was calculated by Eq. (3),

$$188 \quad Q_t = (C_0 - C_t) \times V/m \quad (3)$$

189 where  $Q_t$  ( $\text{mg}\cdot\text{g}^{-1}$ ) was the adsorption capacity of the sorbent for P4 at different time,  $C_0$  ( $\text{mg}\cdot\text{mL}^{-1}$ )  
190 represented the initial concentration of P4 in the solution,  $C_t$  ( $\text{mg}\cdot\text{mL}^{-1}$ ) was the P4 concentration in the  
191 solution at time  $t$ ,  $V$  (mL) was the volume of the working solution, and  $m$  (g) was the weight of the  
192 sorbent.

#### 193 2.5.4 Adsorption selectivity

194 E, E2, DXM, DES and BPA (Fig. 1) were chosen to investigate the selectivity performance of the  
195 CNT@CS/P(MAA). The selectivity of the adsorbent was measured by the selection coefficient  $SC_{P4/R}$ ,  
196 which was calculated by Eq. (4),

$$197 \quad SC_{P4/R} = Q_{P4}/Q_R \quad (4)$$

198 where  $Q_{P4}$  ( $\text{mg}\cdot\text{g}^{-1}$ ) was the adsorption capacity of the adsorbent for P4, and  $Q_R$  ( $\text{mg}\cdot\text{g}^{-1}$ ) was the  
199 adsorption capacity for other reference compounds.

### 200 2.6 SPE procedure

201 A total of 10 mg CNT@CS/P(MAA) was packed in an empty SPE cartridge under room temperature.  
202 After being activated and rinsed by 2 mL of methanol and water successively, the sample solutions were  
203 slowly loaded onto the SPE column. Subsequently, the SPE column was washed with water to remove

204 the water-soluble substances, then the bound targets were eluted with eluting solution. Finally, the eluents  
205 were evaporated with a stream of air. The residues were redissolved in methanol and analyzed by HPLC.

## 206 **2.7 Method validation and real samples analysis**

207 According to the recommendations of the International Conference on Harmonization Q2(R1), the  
208 developed SPE-HPLC system was validated with specificity, linearity, range, limit of detection (LOD),  
209 limit of quantification (LOQ), accuracy and precision.

210 In order to investigate the practicability of the method system, a batch of industrial wastewater  
211 samples were collected from the wastewater treatment system of a steroid hormone pharmaceutical  
212 company in Hanzhong, Shaanxi Province. No. 1 samples were collected in an anaerobic bacteria  
213 treatment tank closed to the industrial production, and No. 2 samples were collected at the end of the  
214 sewage treatment system, which would be discharged directly into the environment. After centrifuged 5  
215 minutes by 4000rpm, those samples were applied to the method system.

## 216 **3. Results and discussion**

### 217 **3.1 Preparation of CNT@CS/P(MAA)**

218 The preparation process of CNT@CS/P(MAA) is shown in Fig. 2. CNT@CS was prepared by  
219 coating chitosan on the surface of CNTs through glutaraldehyde cross-linking. This method could  
220 effectively improve the water-solubility and dispersity of CNTs by introducing hydroxyl and amino  
221 groups on the chitosan layer. During the pre-polymerization process, the carboxyl groups of methacrylic  
222 acid were attracted by the amino groups on the surface of CNT@CS, so the methacrylic acid molecules  
223 were deposited on the carrier surface. Under the heat initiation, MAA polymers with porous structures  
224 were grafted on the surface of CNT@CS through free radical reaction. The CNT@CS/P(MAA)  
225 composites could be combined with P4 through electrostatic interaction (such as hydrogen bonds) and  
226 spatial interaction force. During the polymerization, the amount of MAA and EGDMA could affect the  
227 effective binding sites and the pore size of CNT@CS/P(MAA). Subsequently, the amount of MAA and  
228 EGDMA were optimized.

#### 229 3.1.1 The optimization of MAA

230 In order to explore the optimal adsorption performance of CNT@CS/P(MAA), the amount of MAA  
231 was studied. As shown in Table S1, the results of CNT@CS/P(MAA) exhibit the highest adsorption  
232 capacity, when the mass ratio of carrier/MAA is 4:1. This is because when the amount of MAA is  
233 insufficient, sufficient binding sites cannot be provided. On the contrary, when the amount of MAA is  
234 too much, the binding sites will form competitive adsorption, which will also reduce the adsorption  
235 performance.

#### 236 3.1.2 The optimization of EGDMA

237 The amount of EGDMA was important to control the pore size of the polymers, as well as the  
238 performance of the functional composites. Adsorption performance of the polymers with different  
239 amount of EGDMA are investigated, as shown in the Table S2. As the increases of the amount of  
240 EGDMA, the adsorption capacity of the material decreases gradually, while the selectivity performance  
241 shows the opposite phenomenon. When the amount of EGDMA increases, the degree of crosslinking of  
242 the polymers increases. As a result, the structure of the polymers becomes more denser and the cavities  
243 carried are smaller, which makes it more difficult for the analytes to enter and exit the pores freely.



244 Therefore, the adsorption property of the polymers to P4 is reduced. However, the functional groups and  
245 three-dimensional structure of P4 analogues are different from P4. When the polymers structure becomes  
246 denser, it is more difficult for P4 analogues to pass through the pores and bind to the sites of action. Thus,  
247 the selectivity of the polymers increases. As shown in Table S2, when the EGDMA dosage is 50 mg, the  
248 value of  $SC_{P4/E}$  is maximum. Therefore, 50 mg of EGDMA was selected as the final amount.

### 249 **3.2 Characterization of CNT@CS/P(MAA)**

#### 250 3.2.1 TEM analysis

251 As shown in Fig. 3, the morphology of each material is observed by TEM. Multi-walled carbon  
252 nanotubes (Fig. 3A) have a smooth surface and good light transmittance. Compared with CNTs, the light  
253 transmittance of CNT@CS (Fig. 3B) is decreased and the surface of CNT@CS is slightly rough,  
254 demonstrating that the CS layer has been modified on the surface of the carbon nanotubes. As for  
255 CNT@CS/P(MAA) (Fig. 3C), the light transmittance is further decreased, and it is observed that obvious  
256 coarse-grained polymers are attached to the surface of the carbon nano-materials, suggesting that the  
257 MAA polymers have been grafted onto the surface of CNT@CS.

#### 258 3.2.2 FT-IR spectra analysis

259 FT-IR spectra of CNTs, CNT@CS and CNT@CS/P(MAA) are shown in Fig. 4. In Fig. 4A, the  
260 characteristic peaks of CNTs appearing at 2115, 2016 and 1980  $\text{cm}^{-1}$  are attributed to the stretching  
261 vibration of carbon-carbon single and double bonds. In Fig. 4B, the absorbance peaks at 3340, 1389,  
262 1034 and 875  $\text{cm}^{-1}$ , correspond to the stretching and bending vibration of N–H, O–H, and C–O–C,  
263 respectively, indicating that the CS layer is coated on the surface of the CNTs. In Fig. 4C, the peaks at  
264 2980 and 2789  $\text{cm}^{-1}$  (C–H bond stretching vibration), 1465  $\text{cm}^{-1}$  (C-H bond bending vibration), 1106,  
265 1080 and 1030  $\text{cm}^{-1}$  (C–C bond stretching vibration), 1630 and 1389  $\text{cm}^{-1}$  (C=O bond stretching  
266 vibration) appear, while the peaks at 2115, 2016 and 1980  $\text{cm}^{-1}$  are rapidly decreased, revealing that  
267 methacrylate polymers has been wrapped onto the surface of CNTs@CS, which is consistent with the  
268 TEM results.

#### 269 3.2.3 XRD analysis

270 The crystal structure of the CNTs, CNT@CS and CNT@CS/P(MAA) were characterized by XRD.  
271 As shown in Fig. S1, the CNT@CS and CNT@CS/P(MAA) exhibit the same XRD signals at (002),  
272 (100), (101) and (004) as with CNTs. The diffraction peaks at (002) and (004) are the characteristic  
273 diffraction peaks of carbon tubes, which matched well with the database of carbon nanotubes in JCPDS  
274 (JCPDS card: 02–0456). The diffraction peak at (100) and (101) was the diffraction peak of high-phase  
275 graphite. The CNT, CNT@CS and CNTS@CS/P (MAA) all showed consistent X-ray diffraction peaks,  
276 indicating that the crystal structure of the carbon nanotube carriers is not affected by the grafted chitosan  
277 layer and methacrylic polymer.

#### 278 3.2.4 Nitrogen adsorption-desorption of CNTs and CNT@CS/P(MAA)

279 Nitrogen sorption isotherms (Fig. S2) of CNTs and CNT@CS/P(MAA) samples showed similar II  
280 isotherm, implying a fast nitrogen gas uptake at relative pressures < 0.10 and multi-molecular layer  
281 adsorption of nitrogen. Table 1 shows that the BET surface area of CNT@CS/P(MAA) sample (87.33  
282  $\text{m}^2\cdot\text{g}^{-1}$ ) is obviously lower than that of CNTs (121.44  $\text{m}^2\cdot\text{g}^{-1}$ ) due to the adhesion of methacrylate  
283 polymers layer. While the total pore volume and average pore diameter of the CNT@CS/P(MAA) (0.47

284  $\text{cm}^3\cdot\text{g}^{-1}$  and  $216.82 \text{ \AA}$ , respectively) are higher than these of CNTs ( $0.40 \text{ cm}^3\cdot\text{g}^{-1}$  and  $132.50 \text{ \AA}$ ,  
285 respectively), meaning that CNT@CS/P(MAA) has more mesoporous structures than CNTs. Therefore,  
286 CNT@CS/P(MAA) could provide more accessible cavities and binding sites for target analytes, making  
287 it easier for the targets to enter the identification cavities.

### 288 3.2.5 Thermogravimetric analysis

289 TGA analysis of the CNTs and CNT@CS/P(MAA) were also performed. As shown in Fig. S3, two  
290 main weight losses are observed for the two materials. The first weight loss under  $100 \text{ }^\circ\text{C}$  arise from the  
291 volatilization of moisture, where the weight loss of the CNT@CS/P(MAA) is the same as the CNTs. The  
292 second weight loss of CNT@CS/P(MAA) over  $300 \text{ }^\circ\text{C}$  is significantly higher than that of CNTs,  
293 especially at  $300\text{-}500 \text{ }^\circ\text{C}$ . Unlike bare CNTs, CNT@CS/P(MAA) is coated with a chitosan layer and a  
294 methacrylate polymer layer, indicating that the weight loss at  $300\text{-}500 \text{ }^\circ\text{C}$  was a result of chitosan and  
295 polymers decomposition. At  $500\text{-}800 \text{ }^\circ\text{C}$ , the CNTs and CNT@CS/P(MAA) have the same rate of weight  
296 loss due to the decomposition and oxidation of the carbon phase. The results are agreed with the published  
297 report (Hua et al. 2018). The CNT@CS/P(MAA) is stable at temperatures up to  $300 \text{ }^\circ\text{C}$ , which indicates  
298 its suitability for routine analysis.

## 299 3.3 Adsorption properties

### 300 3.3.1. Optimization of adsorption conditions

301 Adsorption experiments were performed in a methanol-water solution with different water contents,  
302 pH values and temperature. The results are shown in Fig. S4. The adsorption capacity of  
303 CNTs@CS/P(MAA) for P4 was increased with the increase of water content (Fig. S4(A)). When the  
304 water content increase, the hydrophobic force increase, which drives the analytes into the pores of the  
305 material and interacts with the active sites. However, when the water content is too much (over 80%),  
306 the progesterone crystals would precipitate out at the concentration of  $50 \mu\text{g}\cdot\text{mL}^{-1}$ . Therefore, methanol:  
307 water (2:8, V/V) solution is selected as the adsorption solvent in the subsequent experiments.

308 Moreover, the influence of the pH value and the temperature on adsorption capacity were  
309 investigated as shown in Fig. S4(B) and Fig. S4(C). When the pH value changed from 3.0 to 11.0, the  
310 adsorption capacity of CNT@CS/P(MAA) for P4 did not change significantly, indicating that the  
311 adsorption capacity of the prepared materials for P4 was not affected by the pH value of aqueous phase.  
312 At the same time, the adsorption temperatures had little effect on the adsorption property of the prepared  
313 materials. The CNT@CS/P(MAA) has a very stable specific adsorption performance for P4 at  
314 temperatures below  $45 \text{ }^\circ\text{C}$  and different pH values. Therefore, it could be used for the enrichment of P4  
315 in the complex industrial wastewater samples.

### 316 3.3.2 Adsorption isotherms

317 The adsorption isotherms of CNT@CS/P(MAA) for P4 are shown in Fig. 5. Under different  
318 temperatures, the isothermal adsorption curves of CNT@CS/P(MAA) are almost the same. The  
319 adsorption capacity of CNT@CS/P(MAA) is significantly increased with increasing initial concentration  
320 of P4, and do not reach equilibrium even though the concentration of P4 is saturated at  $175 \text{ mg}\cdot\text{L}^{-1}$ . At  
321 the concentration of  $175 \text{ mg}\cdot\text{L}^{-1}$ , the maximum adsorption capacity of CNT@CS/P(MAA) to P4 is  $44.45$   
322  $\text{mg}\cdot\text{g}^{-1}$ , which is higher than that reported in the literatures (Hao et al. 2015, Li et al. 2020, Zheng et al.  
323 2018). To further verify the binding properties of CNT@CS/P(MAA), the Langmuir model and

324 Freundlich model were applied to fit the data, and relevant parameters were calculated. Two equations  
325 are expressed by Eq. (5) and Eq. (6), respectively.

$$326 \quad C_e/Q_e = C_e/Q_m + 1/Q_m \times K_1 \quad (5)$$

$$327 \quad \ln Q_e = \ln C_e/n + \ln K_f \quad (6)$$

328 where  $Q_e$  ( $\text{mg}\cdot\text{g}^{-1}$ ) is the adsorption capacity at equilibrium;  $C_e$  ( $\text{mg}\cdot\text{L}^{-1}$ ) is the equilibrium concentration  
329 of P4;  $K_1$  and  $Q_m$  are the Langmuir constant and the maximum theoretical adsorption capacity,  
330 respectively;  $K_f$  and  $n$  are the Freundlich constant and heterogeneity factor, respectively.

331 As shown in Table 2, the correlation coefficients of Freundlich model were higher ( $R^2 \geq 0.9645$ )  
332 than those of Langmuir model ( $R^2 \leq 0.9095$ ) under different temperatures. This signified that the  
333 Freundlich model could more accurately described the isotherm data of CNT@CS/P(MAA). The  
334 Freundlich isotherm model assumes a multilayer adsorption occurring on a heterogeneous surface, and  
335 the heat of adsorption is not uniform between the molecules adsorbed onto the surface of adsorbent.  
336 Therefore, the adsorption process of CNT@CS/P(MAA) for P4 was multilayer adsorption behavior, or  
337 the adsorption of P4 occurs on a heterogeneous interface between the solution and CNT@CS/P(MAA).

### 338 3.3.3 Adsorption kinetics

339 Fig. 6 shows the kinetic curve of CNT@CS/P(MAA) for P4 at 25 °C. It is obvious that the  
340 adsorption capacity increases rapidly in the first few minutes and reaches the adsorption equilibrium  
341 within 60 minutes. The saturation adsorption capacity of CNT@CS/P(MAA) is  $18.98 \text{ mg}\cdot\text{g}^{-1}$  at the  
342 concentration of  $50 \mu\text{g}\cdot\text{mL}^{-1}$ . Then the pseudo-first-order rate Eq. (7) and pseudo-second-order rate Eq.  
343 (8) are used to fit the kinetic data, and the results are showed in Table 3.

$$344 \quad \lg Q_e/Q_t = \lg Q_e - k_1 \times t/2.303 \quad (7)$$

$$345 \quad t/Q_t = 1/(k_2 \times Q_e^2) + t/Q_e \quad (8)$$

346 where  $k_1$  and  $k_2$  are the adsorption rate constants of the pseudo-first-order equation and pseudo-second-  
347 order equation, respectively;  $Q_e$  ( $\text{mg}\cdot\text{g}^{-1}$ ) is the adsorption capacity at equilibrium, and  $Q_t$  ( $\text{mg}\cdot\text{g}^{-1}$ ) is the  
348 adsorption capacity at time  $t$ .

349 As shown in Table 3, compared with the pseudo-first-order kinetic model, the regression correlation  
350 coefficient of the pseudo-second-order kinetic model ( $R^2 = 0.9745$ ) is higher. The theoretical maximum  
351 adsorption capacity  $Q_e$  calculated by the pseudo-second-order model ( $18.60 \text{ mg}\cdot\text{g}^{-1}$ ) is closer to the  
352 experimental value ( $18.98 \text{ mg}\cdot\text{g}^{-1}$ ). The results demonstrated that the adsorption of P4 onto  
353 CNT@CS/P(MAA) followed the pseudo-second-order kinetic model. Hence, the adsorption rate is  
354 limited by chemisorption which involved the electron sharing or transfer between CNT@CS/P(MAA)  
355 and P4.

### 356 3.3.4 Adsorption selectivity

357 E, E2, DXM, DES and BPA (Fig. 1) were chosen to study the selectivity of the prepared polymers.  
358 The selection coefficient is a quantitative parameter used to evaluate the discrimination ability of  
359 different materials for the target analyte from interfering analogues. The  $SC$  was calculated by Eq. (4)  
360 and the results are shown in Table 4. Compared with CNTs and CNT@CS, the selectivity of  
361 CNT@CS/P(MAA) for P4 is significantly improved. The calculated  $SC$  values for E, E2, DXM, DES  
362 and BPA are 2.42, 2.46, 10.03, 18.94, and 34.43, respectively, indicating the high specificity of  
363 CNT@CS/P(MAA) towards P4. There are differences between P4 and the five analogues in the steroidal

364 ring, three-dimensional structure and functional group, which lead to their different spatial distribution  
365 and different interactions with the active sites in the polymer cavities, resulting in a high selectivity for  
366 P4.

367 The plentiful carboxyl in MAA polymers provided adsorption sites for binding to carbonyl and  
368 hydroxyl groups. Therefore, the adsorption capacity of CNT@CS/P(MAA) mainly depends on the  
369 amount of carboxyl groups and the pore size of the materials surface. P4, E, E2 and DXM contain  
370 carbonyl or hydroxyl groups, which could interact more favorably with the carboxyl in MAA polymers.  
371 Among them, E and E2 have a spatial structure similar to that of P4, and could more freely enter the  
372 holes of the MAA polymers. Moreover, both the C3-carbonyl and C17-methyl ketones of P4 can interact  
373 electrostatically with the carboxyl groups of the MAA polymers, while E and E2 only have C17-carbonyl  
374 or hydroxyl that could interact with the polymers' carboxyl. The force of E and E2 is weaker than that of  
375 P4. DXM is a cortisol hormone. Although it contains two carbonyl groups and three hydroxyl groups,  
376 DXM differs greatly from P4 in spatial structure and could not easily enter the MAA polymers. Therefore,  
377 the selection coefficient of DXM is higher than that of E and E2. DES and BPA are similar to estrogen  
378 in pharmacological action, but their spatial structures and action sites are very different from P4, so the  
379 selection coefficients are higher than others.

#### 380 **3.4 Optimization of SPE conditions**

381 To selectively enrich P4 and remove impurities from water samples, an acid-functionalized  
382 adsorbent was used for SPE, and acetic acid or ammonia were used during the elution to promote the  
383 desorption of P4. The recoveries of P4 under different conditions are shown in Fig 7. The highest  
384 recovery was obtained when the loading volume, elution solvent, and elution volume were 30 mL,  
385 methanol/acetic acid (2:1, v/v), and 3 mL, respectively. As shown in Fig.7A, the recovery slightly  
386 declined when the loading volume of P4 solution exceed 30 mL, because the excessive loading volume  
387 will cause the oversaturation of binding sites. Therefore, a loading volume of 30 mL was selected for  
388 subsequent experiments. The type of eluent also had a great influence on the recovery of P4, as shown  
389 in Fig.7B, the recovery of P4 reach a maximum of 95.3% when the eluent is chloroform/acetic acid (9:1,  
390 v/v). Considering the toxicity of chloroform and the requirements of environmental protection,  
391 methanol/acetic acid (2:1, v/v) with the second highest recovery (82.2%) was chosen as the optimized  
392 eluent. Moreover, the elution volume would affect the recovery of P4. When the volume of eluent is too  
393 small, the target molecules cannot be completely eluted; while the eluent efficiency of impurities will be  
394 increased if the volume of eluent is large enough. As showed in Fig. 7C, when the eluting solution volume  
395 was 3 mL, the recovery of P4 reach the maximum (96.7%). Therefore, 3 mL of the methanol/acetic acid  
396 (2:1, v/v) was selected as the final elution solvent.

#### 397 **3.5 Method validation and application to industrial wastewater samples**

398 The SPE-HPLC method was validated for P4 in tap-water. Fig. 8 shows the chromatograms of P4  
399 standard samples and spiked tap-water samples before and after SPE treatment. It showed that the co-  
400 existing impurities in the tap-water samples without SPE pretreatment could interfere with the detection  
401 of P4. Obviously, the peak areas of the P4 were significantly enhanced after the SPE pretreatment, and  
402 the interference of the impurities were effectively reduced. These results suggest that the established  
403 SPE-HPLC method could effectively eliminate the influence of co-existing impurities in water and enrich

404 the target molecule-P4 in water samples. The linearity of the established analytical method was examined  
405 from calibration curves using a series of spiked samples with concentrations ranging from 0.1 to 600  
406  $\mu\text{g}\cdot\text{L}^{-1}$ . The correlation coefficient of the regression curve was 0.9998, showing a good linearity  
407 relationship.

408 The limit of detection (LOD) of the method was evaluated with a signal-to noise ratio of three (S/N  
409 = 3), and the limit of quantification (LOQ) was determined with a signal-to-noise ratio of ten (S/N = 10).  
410 And the LOD and LOQ were  $3\text{ ng}\cdot\text{L}^{-1}$  and  $10\text{ ng}\cdot\text{L}^{-1}$ , respectively. To test the reusability of the proposed  
411 method, the same SPE column was used to continuously extract P4 in the aqueous phase. The results  
412 (Figure S5) show that the recovery was  $\geq 96.3\%$  after recycling 5 times, indicating that the method had a  
413 good reusability.

414 The accuracy and precision of this method were evaluated by determining the repeatability and  
415 reproducibility of the established method (Table 5). The repeatability and reproducibility were measured  
416 over a five-day period and three SPE columns were used under the same conditions. The accuracy of this  
417 method is ranged from 96.3–104.2%, and the RSD values are less than 5.6%, indicating that the  
418 established SPE-HPLC method has a good accuracy and precision. Therefore, this method could meet  
419 the detection requirements of trace progesterone in the environmental water samples.

420 Then, we detected the dosage of P4 in real industrial wastewater samples using the established  
421 method. In the No. 1 samples collected near the industrial production, the concentration of P4 detected  
422 after SPE pretreatment was  $0.48\text{ mg}\cdot\text{L}^{-1}$ , which was 1.78 times the P4 concentration ( $0.27\text{ mg}\cdot\text{L}^{-1}$ )  
423 without SPE treatment. Meanwhile, in the No. 2 samples collected at the end of the sewage treatment  
424 system,  $0.003\text{mg}\cdot\text{L}^{-1}$  of P4 was detected by the established SPE-HPLC method, while no progesterone  
425 was detected by HPLC without SPE treatment. These results show that even in complex industrial  
426 production wastewater samples, the established SPE-HPLC method could still effectively enrich P4 and  
427 achieve the detection of P4 at a very low concentration. Therefore, the proposed method is suitable for  
428 the effective enrichment and detection of P4 in the industrial wastewater.

### 429 **3.6 Comparison study**

430 The developed SPE-HPLC method was compared with previously reported methods(Hao et al. 2015,  
431 Li et al. 2020, Lucci et al. 2011, Mirzajani et al. 2019b, Razmkhah et al. 2018, Zhang et al. 2020a, Zheng  
432 et al. 2018). The results are listed in Table 6. Compared with other methods, the developed SPE-HPLC  
433 method exhibited excellent adsorption capacity, outstanding sensitivity and relatively low detection  
434 limits. This method also provided a new strategy for the detection of steroid hormones in the wastewater  
435 and environmental samples.

### 436 **4. Conclusions**

437 In this study, CNT@CS/P(MAA) were fabricated by using a simple procedure, and served as novel  
438 extraction sorbents for the specific enrichment of P4 in the industrial wastewater samples. The results  
439 demonstrated that the fabricated carbon materials exhibited excellent adsorption capacity, high  
440 selectivity and prominent sensitivity, and was almost unaffected by the ambient temperature and pH  
441 value. The high selectivity of CNT@CS/P(MAA) for P4 was mainly due to the steric effect and hydrogen  
442 bonding force between them. Additionally, the method validation showed a good linearity, satisfactory  
443 recoveries and stable repeatability. The established method could effectively enrich and detect P4 in the

444 actual industrial wastewater samples without the interference of matrix complexity. Compared with the  
445 published reports, the proposed method has the characteristics of large adsorption capacity, stable  
446 adsorption performance and high sensitivity. It could be a promising alternative for P4 monitoring in the  
447 industrial wastewater, and provides a new method for the detection of P4 in other environmental waters.  
448

449 **Declarations:**

450 **Ethics approval and consent to participate**

451 Not applicable

452 **Consent for publication**

453 Not applicable

454 **Availability of data and materials**

455 All data generated or analysed during this study are included in this published article and its  
456 supplementary information files.

457 **Competing interests**

458 The authors declare that they have no known competing financial interests or personal  
459 relationships that could have appeared to influence the work reported in this paper.

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463 **Authors' contributions**

464 Xia Cui ([cuixia0415@stu.xjtu.edu.cn](mailto:cuixia0415@stu.xjtu.edu.cn)): Conceptualization, methodology, formal analysis, investigation,  
465 visualization, writing—original draft

466 Hua Shu ([shuhuas@stu.xjtu.edu.cn](mailto:shuhuas@stu.xjtu.edu.cn)): Methodology, formal analysis, writing—review and editing

467 Lu Wang ([wanglu0914@mail.xjtu.edu.cn](mailto:wanglu0914@mail.xjtu.edu.cn)): Writing—review and editing

468 Guoning Chen ([chengn1992@stu.xjtu.edu.cn](mailto:chengn1992@stu.xjtu.edu.cn)): Writing—review and editing

469 Jili Han ([HJL1995061968@stu.xjtu.edu.cn](mailto:HJL1995061968@stu.xjtu.edu.cn)): Writing—review and editing

470 Qianqian Hu ([huqiangqian@stu.xjtu.edu.cn](mailto:huqiangqian@stu.xjtu.edu.cn)): Writing—review and editing

471 Kamran Bashir ([kamran2016@stu.xjtu.edu.cn](mailto:kamran2016@stu.xjtu.edu.cn)): Writing—review and editing

472 Zhimin Luo ([luozm0905@xjtu.edu.cn](mailto:luozm0905@xjtu.edu.cn)): Visualization, writing—review and editing

473 Chun Chang ([changchun@mail.xjtu.edu.cn](mailto:changchun@mail.xjtu.edu.cn)): Supervision, funding acquisition

474 Jia Zhang ([zadam@sxhjp.com](mailto:zadam@sxhjp.com)): Funding acquisition

475 Qiang Fu ([fuqiang@mail.xjtu.edu.cn](mailto:fuqiang@mail.xjtu.edu.cn)): Conceptualization, visualization, supervision, funding  
476 acquisition

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481 **Authors' information**

482 **Affiliations**

483 **Department of Pharmaceutical Analysis, School of Pharmacy, Xi'an Jiaotong University, Xi'an**  
484 **710061, China**

485 Xia Cui, Hua Shu, Lu Wang, Guoning Chen, Jili Han, Qianqian Hu, Kamran Bashir, Zhimin Luo, Chun  
486 Chang, Qiang Fu.

487 **Institute of Drug Safety and Monitoring, Academy of Pharmaceutical Science and Technology,**  
488 **Xi'an Jiaotong University, Xi'an 710061, China**

489 Xia Cui, Hua Shu, Lu Wang, Guoning Chen, Jili Han, Qianqian Hu, Kamran Bashir, Zhimin Luo, Chun  
490 Chang, Qiang Fu.

491 **Shaanxi Hanzhong Pharmaceutical Group Co., Ltd, Hanzhong 723000, China**

492 Jia Zhang, Qiang Fu.

493

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