**Determination of trace antibiotics in water and milk via preconcentration and cleanup using activated carbons**

# Determination of trace antibiotics in water and milk via preconcentration and cleanup using activated carbon

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Abbreviations: ABX, antibiotics; SMM, sulfamonomethoxine sodium; OTC, oxytetracycline; CEF, ceftiofur hydrochloride; MAR, marbofloxacin; MRLs, maximum residue limits; HPLC, high pressure liquid chromatography; SPE, solid phase extraction; RE, recovery efficiency; ACs, activated carbons; CPAC, coconut powdered AC; $S_{BET}$, Brunauer-Emmett-Teller surface area; $V_{Meso}$, mesopore volume; $V_{Micro}$, micropore volume; PGAC, peat granular AC; WPAC, wood powder AC; MeCN, acetonitrile; TFA, trifluoroacetic acid; MeOH, methanol; EtOH, ethanol; $NH_4OH$, ammonia solution; EDTA, ethylenediaminetetraacetic acid disodium salt hydrate; t-BuOH, t-butanol; n-PrOH, n-propanol; MW, molecular weight; MV, molar volume; Log$K_{OW}$, octanol-water partition coefficient; $S_{Water}$, solubility of ABX in water; $S_{EtOH}$, saturated mole fraction solubility of ABX in EtOH; p$K_a$, dissociation constant; NHA, number of H-bond acceptors; NHD, number of H-bond donors; Refs., references; *Bp*, boiling point; $\varepsilon$, dielectric constant; EDA, $\pi$–$\pi$ electron donor-acceptor; TC, tetracycline; LODs, limit of detections; LOQs, limit of quantitations; LRs, linear ranges; RSD, relative standard deviation; FA, formic acid; DCM, methylene chloride; MSPD, magnetic solid phase dispersion; MSPE, magnetic solid phase extraction.

**Abstract**

CPAC-SPE-HPLC (coconut powdered activated carbon -SPE- HPLC) has been developed for the determination of antibiotic (ABX), sulfamonomethoxine sodium (SMM), oxytetracycline (OTC), ceftiofur hydrochloride (CEF) and marbofloxacin (MAR), in water and milk. Over 99.0% SMM and OTC were recovered from 20 mL of 0.5 μg/mL ABX solution using 10 mg-CPAC for adsorption and 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) for elution. Similarly, over 99.0% CEF and MAR were recovered using 15 mg-CPAC and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v). Moreover, the *REs* of various ABX from 5-80 mL of 0.02-2.00 μg/mL medicated milk containing 10 mM EDTA are ordered as follows: OTC (99.3%), SMM (99.1%) > CEF (68.9%) > MAR (61.4%). No interference towards HPLC analysis were observed with elution using 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v). Furthermore, much lower limit of detections (0.02 μg/mL) than the maximum residual limits from European Commission (0.075-0.100 μg/mL) were obtained.

**Keywords:** *Antibiotic residues in milk; Preconcentration; Cleanup; Activated carbon; Solid phase extraction.*

## 1. Introduction

Antibiotics (ABX) sulfamonomethoxine sodium (SMM), oxytetracycline (OTC), ceftiofur hydrochloride (CEF) and marbofloxacin (MAR), etc. are currently frequently used in European dairy farms, and their residues in milk are cause for serious concern in Europe. The European Commission has adopted maximum residue limits (MRLs) of SMM (100 μg/L), OTC (100 μg/L), CEF (100 μg/L), and MAR (75 μg/L) in milk to guarantee the safety of dairy products and the health of human beings [1]. Thus, the development of QuEChERS (quick, easy, cheap, effective, rugged and safe) methods for determining the levels of trace ABX in milk is important for dairy farmers, milk processors, regulatory authorities and researchers [2,3].

The methods for determining trace ABX in milk include chromatographic methods, microbiological approaches, immunochemical techniques and biosensors, etc. [2–5], with high pressure liquid chromatography (HPLC) seeing extensive use. As milk contains complex ingredients, such as protein, lactose, fat and inorganic ions, the determination of ABX can be disturbed by milk ingredients during HPLC analysis [6]. This means that sample pretreatment is the most critical procedure as it can eliminate interference from milk ingredients (cleanup) and enrich ABX in magnitude (preconcentration) in order to achieve accurate ABX determination [2,7,8]. Several pretreatment methods, including solvent extraction, solid phase extraction (SPE) and centrifugal ultrafiltration, etc., have been developed [2,9,10]. SPE is an updated pretreatment method that is based on low-pressure liquid chromatography and liquid-solid phase equilibrium, and has been widely used to separate target analytes from

76 samples, preconcentrate ABX and clean samples for HPLC analyses [2,11]. Compared
77 to solvent extraction, SPE is more convenient and efficient. However, deproteinization
78 and the follow-up of centrifugation, sample loading, evaporation, reconstitution and
79 filtration are still necessary [10]. If the deproteinization procedure of milk is skipped,
80 high throughput of milk in the SPE column can cause blockage, an unstable flowrate
81 and inconsistent adsorption times. In addition, either MeOH or $H_2O$ are usually used to
82 clean up SPE columns after sample loading to further eliminate the interference of
83 impurities [5,6,11].

84 Adsorption efficiency is directly associated with the recovery efficiency (*RE*) of
85 ABX when using SPE. Various adsorbents, such as molecularly imprinted polymers,
86 metal-organic frameworks and carbon materials, etc. have been developed for milk
87 pretreatment [2,8,12]. Of these, activated carbons (ACs) besides the low cost, high
88 adsorption capacity and recyclability, it shows the widest availability. ACs are ideal
89 adsorbents as they also possess distinctive physicochemical properties, such as rich
90 mesopores, high specific surface areas and a variety of functional groups [13,14]. The
91 ABX in milk can be adsorbed onto ACs via physical interactions, such as electrostatic
92 interactions, π-π bonds, H-bonds, etc., and the loaded ABX on the ACs can then be
93 eluted by different eluents [15]. Interestingly, mesoporous ACs belong to the group of
94 “restricted access materials”. The narrow pore diameter of ACs only allows low
95 molecular weight ABX to enter, while high molecular weight proteins or fats cannot
96 enter mesopores [16]. The procedure of chemical deproteinization can therefore
97 probably be skipped when ACs are used for the recovery of ABX from milk.

Furthermore, chelates that are formed between ABX, metal ions ($Ca^{2+}$ and $Mg^{2+}$) and protein in milk can diminish the adsorption of ABX [2,5,6]. Chelating agents, including EDTA, oxalic acid and citric acid, have been added to milk to promote the recovery of ABX [2,5,6,9].

This study aims to develop an AC-SPE-HPLC approach for the determination of trace SMM, OTC, CEF and MAR in milk. Based on the physicochemical properties of ABX and eluents, such as acidity and alkalinity, dissociation, polarity, H-bonds, etc., the adsorption and elution conditions of the SPE for the recovery of the model ABX from water were optimized. Furthermore, the *REs*, using different ACs, of ABX at various spiked concentrations in milk were evaluated in the absence and presence of EDTA. Finally, the proposed AC-SPE-HPLC method was verified and compared with methods from other published works.

## 2. Materials and Methods

### 2.1. Materials and chemicals

Pasteurized commercial skimmed milk was purchased from a local branch of a major supermarket (Turin, Italy) and stored at room temperature. Preliminary analyses demonstrated that the purchased milk was ABX-free. The main ingredients in 100 mL of milk were carbohydrate (4.8 g), sugar (4.8 g), protein (3.4 g), saturated fat (0.5 g), fatty acid (0.3 g) and salt (0.13 g). The medicated milk samples were prepared daily by adding the stock ABX solutions into the milk. The initial pH of raw, 10 mg/L CEF-, 10 mg/L SMM-, 10 mg/L MAR-, and 10 mg/L OTC-spiked milk samples were determined to be 6.46, 6.56, 6.50, 6.56, and 6.58, respectively.

120 Coconut powdered AC (CPAC) was proven to act as a mesoporous adsorbent in
121 our previous studies [13,15], and was used as the core adsorbent for the pretreatment
122 of milk via SPE in this study. The Brunauer-Emmett-Teller (BET) surface area ($S_{BET}$),
123 mesopore volume ($V_{Meso}$), micropore volume ($V_{Micro}$) and average pore size of CPAC
124 were measured to be 1952 $m^2/g$, 1.57 $cm^3/g$, 1.76 $cm^3/g$ and 3.95 nm, respectively.
125 CPAC was purchased from ACEF S.P.A. Piacenza (Italy). Peat granular AC (PGAC)
126 and wood powder AC (WPAC) were provided by Merck-Sigma-Aldrich, Milan (Italy).
127 The textural properties of PGAC, WPAC and CPAC have been presented in the
128 literature [13].
129 SMM (400 mg/mL, Daimeton 40, IZO Srl), OTC (92.7 mg/mL, Oxtra MV 10,
130 Huvepharma), CEF (50 mg/mL, Ceva Santé Animale), and MAR (100 mg/mL,
131 Vetoquinol) were used as the model ABX in this work. The initial pH of 10 mg/L CEF-,
132 10 mg/L SMM-, 10 mg/L MAR-, and 10 mg/L OTC aqueous solution were determined
133 to be 5.51, 6.67, 5.63, and 6.67, respectively. Their chemical structures, major
134 physicochemical properties and toxicological parameters are shown in Table S1 and in
135 Scheme 1 (in Section 3.1.3), respectively. The information of solvents and water used
136 was listed in the supplementary material (Section S1.0).

137 **2.2. Apparatus and instrument**

138 The analysis of water and milk samples was performed using a range of apparatus,
139 including a high-speed refrigerated centrifuge (Allegra® 64R, Beckman Coulter, US),
140 a UV-Vis spectrometer (Cary 60 UV–vis Spectrophotometer, USA), a HPLC system
141 (Waters Corp., Milford, MA, USA) coupled with a diode array detector (UV/DAD,

142 Waters Corp., Milford, MA, USA), an automatic sampler (Waters Corp., Milford, MA,
143 USA) and a binary HPLC pump (Waters Corp., Milford, MA, USA). An Xterra RP 18
144 separation column (5 μm, 150 mm×4.6 mm; Phenomenex, Torrance, CA, USA) was
145 used with gradient elution and UV-DAD acquisition.

146 The maximum absorption wavelengths for the UV-Vis analysis of SMM, OTC,
147 CEF and MAR in water were found to be 273, 267, 288 and 293 nm, respectively. The
148 HPLC-DAD analysis of the model ABX was performed according to the methods
149 described in Table S2. Briefly, the Xterra RP 18 separation column was pre-equilibrated
150 for 21 min with 0.1%TFA in $H_2O$ (Phase A) and 0.1%TFA in MeCN (Phase B). A needle
151 installed onto an automatic sampler, used for injections, was auto-washed with
152 MeOH/$H_2O$ (1/1 v/v) before each sample injection. The injection volume was set as 20
153 μL. The samples were loaded and analyzed using HPLC-DAD at room temperature,
154 and the flowrate of the mobile phase was set at 0.8 mL/min. The total running time for
155 the analysis of the model ABX ranged from 26 to 32 min. After the analysis of a batch
156 of samples, the separation column was washed with $H_2O$ (phase A) and MeCN (phase
157 B) for 50 min. The identification of ABX was performed by comparing the UV
158 absorption spectra and retention times of the milk samples and standard solutions.

159 **2.3. Preconcentration and cleanup of milk samples**

160 The pretreatment procedures, namely, the preconcentration and cleanup of trace
161 ABX in milk for HPLC analysis, are shown in Fig. 1.

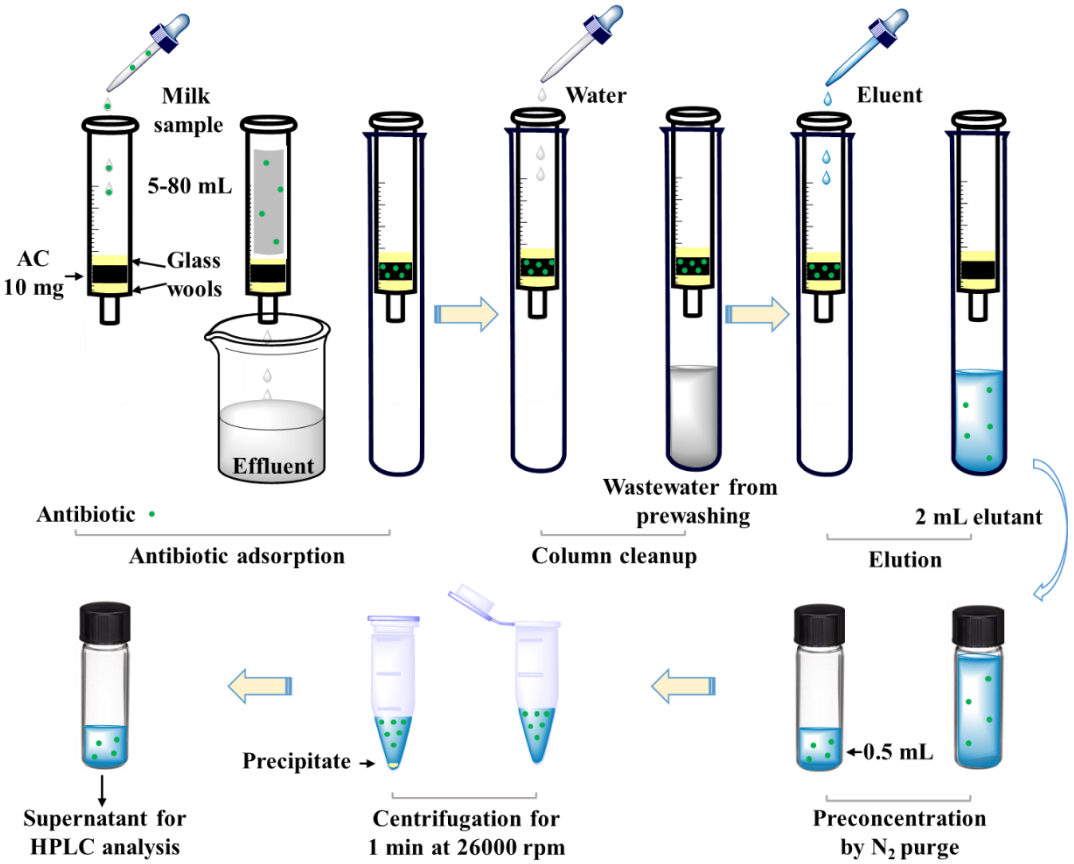



Fig. 1. Procedures of preconcentration and cleanup for the HPLC analysis of trace ABX in milk.

It is necessary to note that different amounts of EDTA had previously been added to the 5-80 mL milk samples so that 10 mM EDTA was contained in each. These were then shaken for 5 min to ensure that the EDTA adequately interacted with the milk matrix and that the milk flowed smoothly in the SPE column [2,5,6,9].

The follow-up procedures were conducted as follows. Firstly, either 10 mg powder AC or 50 mg granular AC were placed into the barrel of a glass syringe (i.d. 1.5 cm), and glass wool was fitted above and below the AC layer to prevent the AC washing out. Secondly, the 5-80 mL medicated-milk samples were passed through the AC column continuously. As a result, trace ABX were absorbed onto the AC and the white milk flowed out from the AC column. The AC column was then flushed, using 1 mL of distilled water, to remove the small amount of milk that adhered to the AC, and the

small amount of water in the AC-column was extruded out. Afterwards, 2 mL of eluent was passed through the ABX-loaded AC column to elute the adsorbed ABX and the elutant was collected in a glass vial. Furthermore, 2 mL of the elutant were concentrated, via mild $N_2$ purge, to 0.5 mL at room temperature. Some insoluble matter was observed and was separated by centrifugation at 26000 rpm for 1 min. Finally, the supernatant was collected and stored at 4 °C for HPLC analysis.

### 2.4. Evaluation of recovery efficiency

The medicated milk samples containing 0.02, 0.10, 0.50 and 1.00 μg/mL ABX were used for the evaluation of the *REs*. The method for preconcentration and cleanup using AC-SPE is described in Section 2.3. *RE* depended on adsorption/desorption efficiency and was calculated in accordance with Eq. (1):

$$RE\ (\%) = \frac{C_d V_d}{C_0 V_0} \times 100\% \qquad (1)$$

where, $C_d$ (μg/mL) is the determined concentration of ABX in the concentrated elutant, $V_d$ (mL) is the volume of the concentrated elutant, $C_0$ (μg/mL) is the spiked concentration of ABX in the milk sample, and $V_0$ (mL) is the volume of the milk sample that passed through the AC-SPE column.

All experiments were repeated at least twice and errors are shown as the difference between the highest measured values and the average value in parallel experiments. When errors are not visible in the figures, they are smaller than the symbols representing the average values.

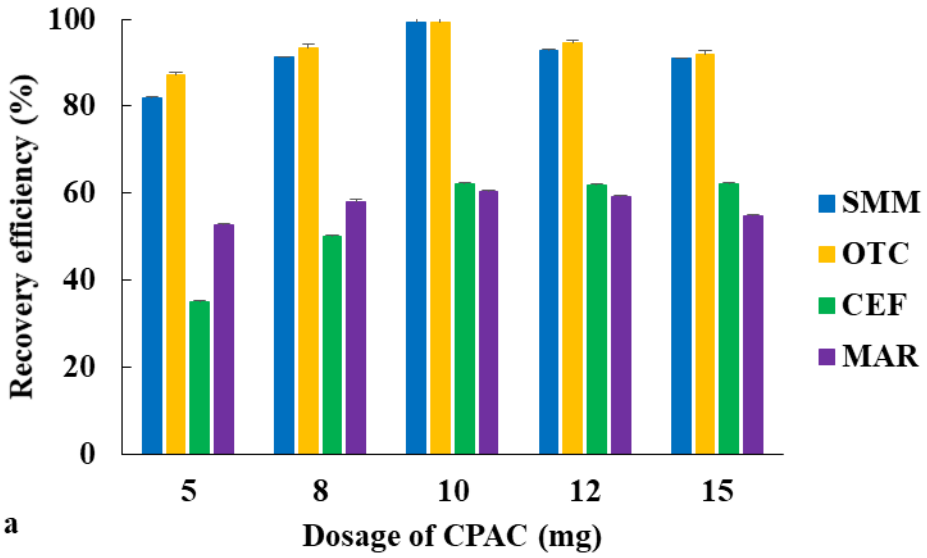
# 3. Results and discussion

## 3.1. Optimization of AC-SPE for ABX recovery from water

The feasibility of using ACs as an adsorbent in SPE is the primary concern here. Although ACs are undoubtedly superior absorbents, the desorption of organic adsorbates from them is generally considered to be a considerable challenge [17,18]. It was therefore necessary to demonstrate the high *RE* of ABX using ACs in water. CPAC has been proven to provide superior adsorption/desorption performance in our previous studies [13,15]. In the preliminary study, using water samples instead of milk samples made the whole AC-SPE operation easier, more economical and more environmentally friendly. The effects of CPAC amount, the flowrate of the water sample, the type, volume and flowrate of the eluent and the use of various ABX were evaluated in the search of optimal conditions on the *REs*.

### 3.1.1. Optimization of adsorption conditions

Both the amount of adsorbent and the flowrate of the water sample through the SPE column are critical factors that influence the *RE* [8,19]. Firstly, the *REs* of the model ABX were evaluated with various doses of CPAC (5-15 mg). 20 mL of 0.5 μg/mL aqueous solutions of ABX were passed through the CPAC-columns at a flowrate of 1.00 mL/min (Adsorption) and 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were passed through the CPAC-columns at a flowrate of 0.07 mL/min (Elution) at room temperature, and the results are presented in Fig. 2a.



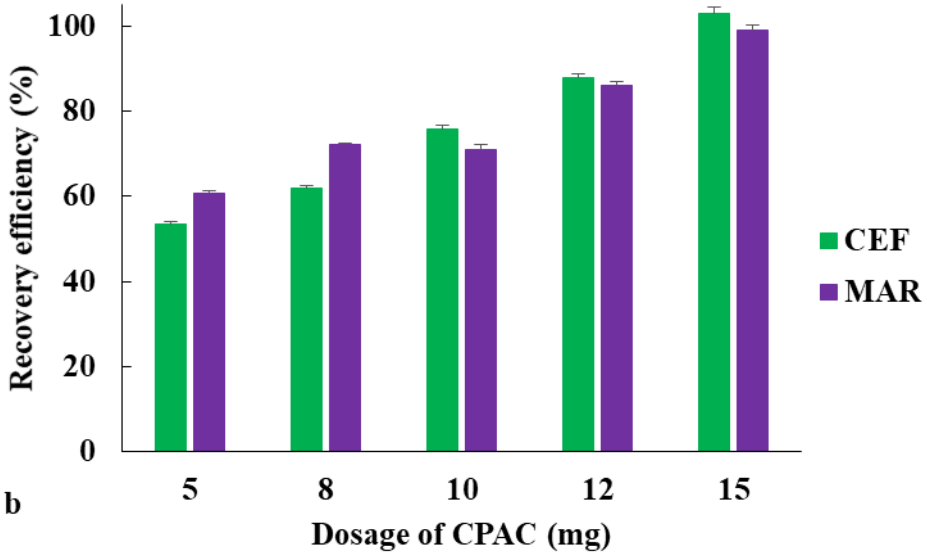




Fig. 2. Effect of CPAC amount on *REs* of ABX in water. (a) *REs* using various amount of CPAC and 30% $NH_4OH$/EtOH (1/19 v/v) at room temperature (Adsorption conditions: 20 mL of 0.5 μg/mL ABX were passed through CPAC columns at 1.00 mL/min. Elution conditions: 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were passed through the ABX-loaded columns at 0.07 mL/min); (b) *REs* of MAR and CEF using various amount of CPAC and 30% $NH_4OH$/n-PrOH (1/19 v/v) at room temperature (Adsorption conditions: 20 mL of 0.5 μg/mL ABX were passed through CPAC columns at 1.00 mL/min. Elution conditions: 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) were passed through the ABX-loaded columns at 0.07 mL/min).

As shown in Fig. 2a, the *REs* of SMM and OTC are much higher than those of CEF and MAR. The reason will be discussed in Section 3.1.3. In addition, increasing CPAC amount initially increases the *REs*, the peak *REs*, 99.4% (SMM), 99.2% (OTC),

227 62.1% (CEF) and 60.4% (MAR), are achieved with 10 mg of CPAC, and then the *REs*
228 decrease slowly with increasing the amount of CPAC. Generally, the increased amount
229 of adsorbent provided more adsorption sites, which ensured that ABX was efficiently
230 adsorbed. However, it is difficult to elute ABX at the higher adsorbent amount (> 10
231 mg of CPAC). The effects of eluent type and amount are discussed in Section 3.1.2.

232 To further increase the *REs* of CEF and MAR, the effect of CPAC amount on the
233 *REs* of CEF and MAR with elution using 30% $NH_4OH$/n-PrOH (1/19 v/v) was further
234 investigated. 20 mL of 0.5 μg/mL aqueous solutions of CEF and MAR were passed
235 through the 5-15 mg CPAC-columns at a flowrate of 1.00 mL/min at room temperature,
236 and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) were used for the elution. The results are
237 shown in Fig. 2b. Obviously, the *REs* of CEF and MAR increase with increasing of
238 CPAC amount and the highest *REs* reach 102.9% for CEF and 99.1% for MAR with 15
239 mg of CPAC. As compared with elution using 30% $NH_4OH$/EtOH (1/19 v/v), the *REs*
240 of CEF and MAR increase by 66% and 81.2% using 30% $NH_4OH$/n-PrOH (1/19 v/v)
241 as eluent, respectively. Importantly, higher amount of the adsorbent (15 mg of CPAC)
242 are required to fully catch CEF and MAR from water.

243 In addition, the adsorption efficiency of ABX is also dependent on the flowrate of
244 the water sample. For example, 20 mL of 0.5 μg/mL MAR aqueous solutions were
245 passed through the 10 mg CPAC-columns at various water-sample flowrates (0.30-2.00
246 mL/min) at room temperature, and elution with 30% $NH_4OH$/EtOH (1/19 v/v) or 30%
247 $NH_4OH$/n-PrOH (1/19 v/v), and the results are shown in Fig. 3.

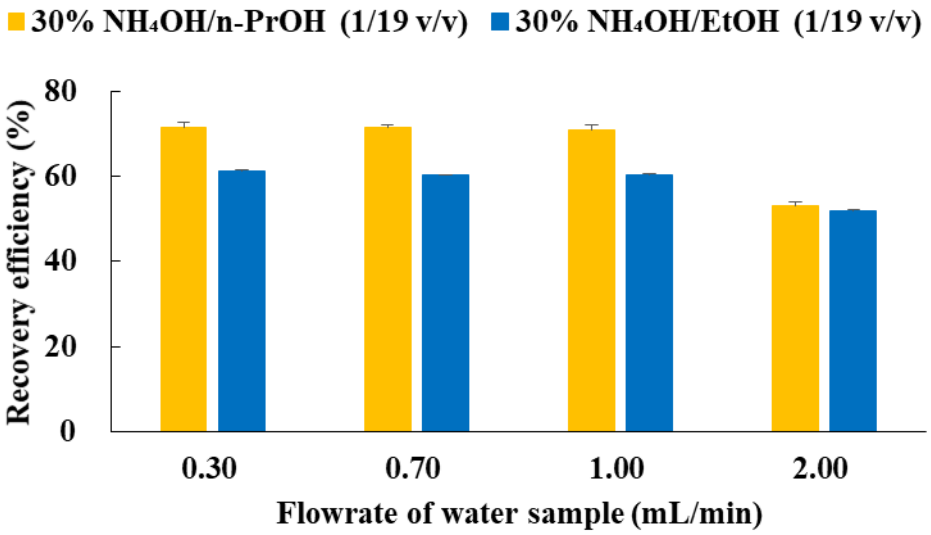



Fig. 3. Effect of water-sample flowrates on the *REs* of MAR at room temperature (Adsorption conditions: 20 mL of 0.5 μg/mL MAR were passed through 10 mg-CPAC columns. Elution conditions: 2 mL of eluents were passed through the MAR-loaded columns at 0.07 mL/min).

As shown in Fig. 3, using 10 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) for elution, no obvious effects of water-sample flowrates between 0.30-1.00 mL/min were observed, but the *REs* of MAR drops from 60.4% to 51.9% as the flowrate increases from 1.00 to 2.00 mL/min. Therefore, the higher sample flowrate results in an insufficient adsorption time and lower *REs* [19]. The similar effects of water-sample flowrates were observed with 10 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) for elution.

The effect of water-sample flowrates on *REs* of MAR with a higher CPAC amount was further studied. With 15 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) for elution, as expected, the *REs* reached 99.1%, 98.1%, 97.7% and 98.2% at the sample flowrates of 0.30, 0.70, 1.00 and 2.00 mL/min, respectively. It indicates that no obvious effects of water-sample flowrate on the *REs* of MAR were observed, 15 mg of CPAC is sufficient for catching trace MAR and 30% $NH_4OH$/n-

PrOH (1/19 v/v) is an ideal eluent.

### 3.1.2. Optimization of elution conditions

The elution conditions, e.g. the type, volume and flowrate of the eluent, determine the selectivity and efficiency of elution [5,8,20]. Acidity and alkalinity, polarity (hydrophobicity, dielectric constant and Log$K_{OW}$ value) and the H-bond interaction (H-donor/acceptor count) of eluents are the critical factors for the elution of organics from ACs [13]. The major physicochemical properties of the eluents are listed in Table 1.

Table 1. Major physicochemical properties of eluents (Data were adopted from Pubchem and Drugbank databases).

| ***Eluent*** | ***MW (g/mol)*** | ***Bp (°C)*** | ***LogK$_{OW}$*** | ***pK$_a$*** | ***NHA*** | ***NHD*** | ***ε*** |
|---|---|---|---|---|---|---|---|
| $H_2O$ | 18 | 100.0 | - | 15.7 | 1 | 1 | 80.4 |
| HOAc | 60 | 117.9 | -0.17 | 4.8 | 2 | 1 | 6.2 |
| $NH_4OH$ | 35 | 38.0 | - | 9.2 | 1 | 2 | 2.5 |
| MeCN | 41 | 81.6 | -0.34 | 8.8 | 1 | 0 | 38.8 |
| MeOH | 32 | 64.7 | -0.77 | 13.1 | 1 | 1 | 32.7 |
| EtOH | 46 | 78.2 | -0.31 | 15.9 | 1 | 1 | 24.6 |
| n-PrOH | 60 | 97.2 | 0.25 | 16.1 | 1 | 1 | 20.3 |
| t-BuOH | 74 | 84.2 | 0.35 | 19.2 | 1 | 1 | 10.9 |
| n-BuOH | 74 | 117.8 | 0.88 | 16.1 | 1 | 1 | 17.5 |

**Note:** MW, molecular weight; *Bp*, boiling point; Log$K_{OW}$, octanol-water partition coefficient; p$K_a$, dissociation constant; NHA, number of H-bond acceptors; NHD, number of H-bond donors; *ε*, dielectric constant.

Referring to our previous work and according to pre-experiments, 30% $NH_4OH$/EtOH (1/19 v/v) was proved to be an ideal eluent for the desorption of organics from CPAC. Based on Fig. 2a, the superior *REs* of SMM and OTC (> 99%) were achieved with elution using 30% $NH_4OH$/EtOH (1/19 v/v), but the *REs* of CEF and MAR were around 60%. In contrast, more than 99% of CEF and MAR were recovered with 15 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) for elution (Fig. 2b). Therefore, CEF and MAR were selected as the model ABX to evaluate

the role of various eluents on their *REs* [8,20]. Typically, 20 mL of 0.5 μg/mL CEF or MAR aqueous solutions were passed through the 10 mg CPAC-columns at a water-sample flowrate of 1.00 mL/min at room temperature. The *REs* of CEF and MAR using various eluents are shown in Fig. 4.

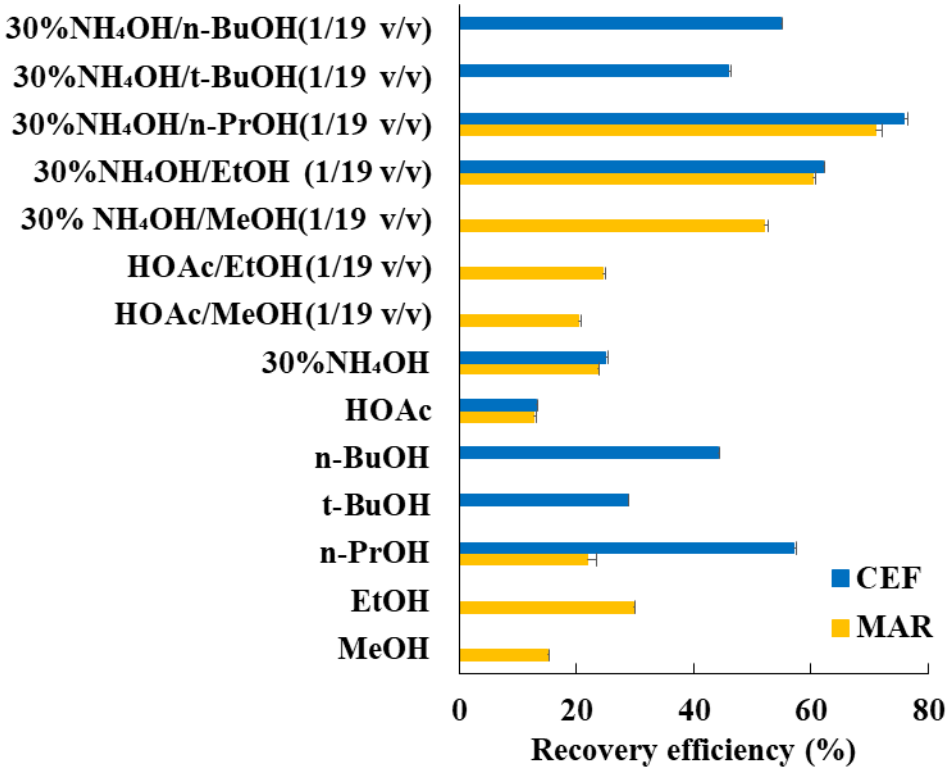



Fig. 4. *REs* of CEF and MAR in water using CPAC columns and various eluents at room temperature (Adsorption conditions: 20 mL of 0.5 μg/mL CEF or MAR were passed through 10 mg-CPAC columns at 1.00 mL/min. Elution conditions: 2 mL of various eluents were passed through the ABX-loaded columns at 0.07 mL/min).

As shown in Fig. 4, remarkable different *REs* of CEF and MAR were observed with elution using various eluents. CEF is a hydrophobic acidic compound with a lower $S_{water}$ (100 mg/L), a higher Log$K_{OW}$ value (1.2), and a lower p$K_a$ value (2.83). In contrast, MAR is a relatively hydrophilic acidic compound with higher $S_{water}$ (2600 mg/L), a lower Log$K_{OW}$ value (-0.53), and a higher p$K_a$ value (5.38), and MAR can be dissolved in EtOH, DMSO, DMF, etc.[21].

Considering the different physicochemical properties of CEF and MAR and the

like-dissolves-like rule, MeOH, EtOH, n-PrOH, t-BuOH or n-BuOH with various polarities were used to elute CEF or MAR from CPAC, respectively (Table 1). The results show that the *REs* of CEF were 57.1%, 44.3% and 28.8% with elution using n-PrOH, n-BuOH and t-BuOH, respectively, while the *REs* of MAR obtained with elution using MeOH, EtOH and n-PrOH reached 15.2%, 29.8% and 22.0%, respectively. In addition, the very polar aprotic or protic solvents, water and MeCN are ineffective for eluting both of CEF and MAR.

The acidic and alkali eluents, HOAc and 30% $NH_4OH$, were evaluated next. HOAc is known as a common carboxylic acid with high polarity and potential for H-bonding with the –COOH, –F or $–NH_2$ groups that exist in CEF or MAR molecules. $NH_4OH$ is a common alkali reagent with high polarity and potential for H-bonding, which favors the desorption of acidic CEF and MAR. The results show that the *REs* obtained only reached 13.2% (CEF) and 12.7% (MAR) with elution using HOAc, as well as 25.1% (CEF) and 23.8% (MAR) with elution using 30% $NH_4OH$, respectively. The effectiveness of 30% $NH_4OH$ is higher than that of HOAc due to the dissociation of –COOH in the CEF and MAR molecules under alkali conditions [22].

To further improve the *REs* of CEF and MAR, acidic and alkali solvents were mixed with alcohols, such as MeOH, EtOH, n-PrOH, t-BuOH or n-BuOH to form co-solvents, so that the acidity and alkalinity, polarity and H-bond interaction of the eluents can synergistically enhance the desorption. As expected, the *REs* of CEF obtained by adding 30% $NH_4OH$ to n-PrOH, t-BuOH, and n-BuOH were significantly increased to 75.8%, 46.0% and 54.9%, respectively. Meanwhile, the *REs* of MAR obtained by

adding 30% $NH_4OH$ to MeOH, EtOH and n-PrOH were extremely increased to 52.0%, 60.4% and 71%, respectively. The more effective elution of CEF and MAR by adding 30% $NH_4OH$ to n-PrOH than other alcohols could be attributed the appropriate polarity of the 30% $NH_4OH$/n-PrOH [8,23,24]. In contrast, the *REs* of MAR obtained using HOAc/MeOH (1/19 v/v) and HOAc/EtOH (1/19 v/v) were 20.5% and 24.5%, respectively, due to the variation of the H-bond interactions and the –COOH dissociation of MAR [8,23,24]. In summary, Lewis bases solvents as eluents exhibit higher desorption efficiency than neutral and Lewis acid solvents [23,24]. The acidity or alkalinity, the polarity and the H-bond interactions of an eluent jointly determined the desorption efficiency and the co-solvent 30% $NH_4OH$/n-PrOH (1/19 v/v) was an optimal eluent.

To further improve the desorption and to minimize the consumption of eluents, the effect of eluent volume on the *REs* of CEF and MAR was studied. In the preliminary study, 20 mL of 0.5 μg/mL MAR were passed through 10 mg-CPAC columns at 1.00 mL/min, and then 1-10 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were passed through the MAR-loaded columns at 0.07 mL/min. As a result, the *REs* of MAR reached 36.6%, 60.4%, 60.75, 61.0%, 61.8 and 64.3% with elution using 1, 2, 4, 6, 8 and 10 mL of the eluent, respectively. The *REs* of MAR increased remarkably as the eluent volume increased from 1 to 2 mL, but only a slight increase in *REs* occurred after 2 mL.

To further understand the effect of eluent volume on the *REs*, 30% $NH_4OH$/n-PrOH (1/19 v/v) was used as eluent. 20 mL of 0.5 μg/mL MAR or CEF were passed through 15 mg-CPAC columns at 1.00 mL/min, and then 1-4 mL of the eluent were passed

through the ABX loaded columns at 0.07 mL/min. The results are reported in Fig. 5a.

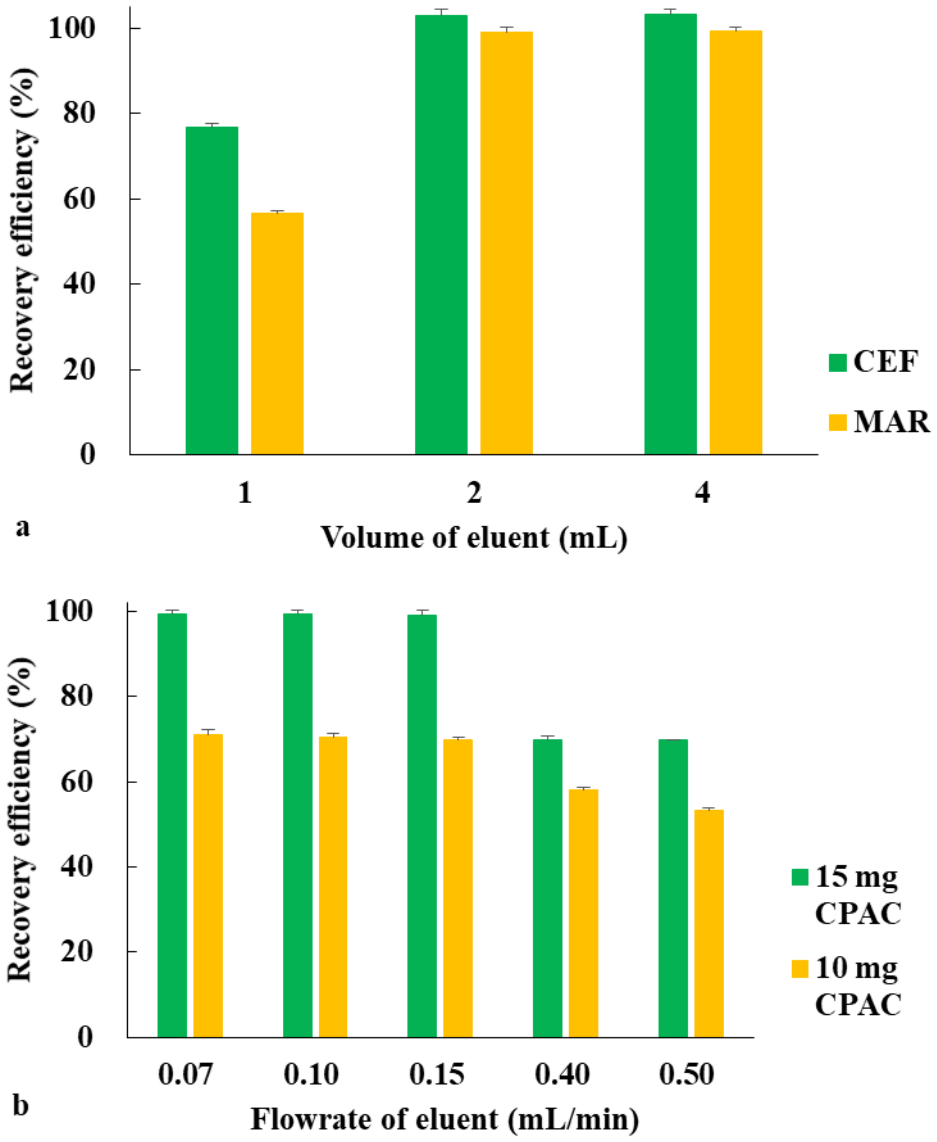



Fig. 5. Optimizing the elution processes of CEF and MAR from CPAC at room temperature. (a) Effect of eluent volumes on the *REs*-of CEF and MAR (Adsorption conditions: 20 mL of 0.5 µg/mL ABX were passed through 15 mg-CPAC columns at 1.00 mL/min. Elution conditions: 1-4 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) were passed through the ABX-loaded columns at 0.07 mL/min); (b) Effect of eluent flowrates on the *REs* of MAR (Adsorption conditions: 20 mL of 0.5 µg/mL MAR were passed through CPAC columns at 1.00 mL/min. Elution conditions: 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) were passed through the MAR-loaded columns at various flowrates).

As shown in Fig. 5a, the *REs* of MAR and CEF increased from 56.6% to 99.1%, and from 76.7% to102.9% as the volume of 30% $NH_4OH$/n-PrOH (1/19 v/v) increased from 1 to 2 mL, respectively, but no changes in the *REs* were observed after 2 mL. It is similar to the effects of the 30% $NH_4OH$/EtOH (1/19 v/v) volume on the *REs* of MAR

355 and previous study's result [25]. The results reveal that 15 mg CPAC is sufficient for
356 full adsorption of CEF and MAR from water, and 2 mL of 30% $NH_4OH$/n-PrOH (1/19
357 v/v) is a better eluent for eluting CEF and MAR from CPAC.

358 Furthermore, the desorption efficiency of ABX is also dependent on the flowrate
359 of eluents [26]. The *REs* of MAR from loaded CPAC columns were evaluated at various
360 flowrates of 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) at room temperature, and the
361 results are shown in Fig. 5b. Using 10 or 15 mg of CPAC for adsorption and 2 mL of
362 30% $NH_4OH$/n-PrOH (1/19 v/v) for elution, no obvious effects of eluent flowrates
363 between 0.07-0.15 mL/min were observed, but *REs* of MAR significantly drops as the
364 flowrate increases from 0.15 to 0.50 mL/min (Fig. 5b). Obviously, a higher eluent
365 flowrate results in an insufficient desorption time and lower *REs* [26]. Consequently,
366 0.15 mL/min was proven to be the optimal flowrate for eluting MAR from CPAC.

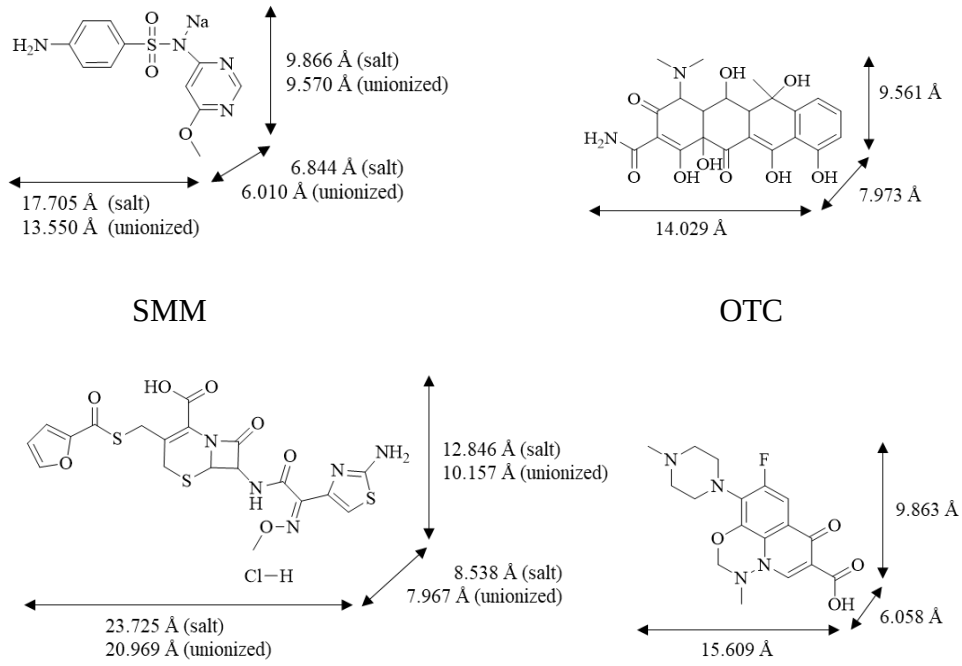
367 **3.1.3. Recovery mechanism of ABX from water**

368 The *REs* of ABX are different despite the same analysis method being used as they
369 all have different chemicals structures and physicochemical properties [2,5]. As shown
370 in Fig. 2a, the *REs* of SMM and OTC are much higher than those of CEF and MAR,
371 suggesting that 10 mg-CPAC as an absorbent and 2 mL of 30% $NH_4OH$/EtOH (1/19
372 v/v) as an eluent are highly efficient for the recovery of trace SMM and OTC. In contrast,
373 the *REs* of CEF and MAR reached 102.9% and 99.1%, respectively, as 15 mg CPAC
374 and 2 mL of 30% $NH_4OH$/n-PrOH (1/19 v/v) were used (Fig. 2b).

375 Generally, the $S_{BET}$, $V_{Meso}$ and $V_{Meso}$ dominate the adsorption performance of ACs
376 [17,27,28]. As mentioned in Section 2.1, CPAC possesses a very large $S_{BET}$ and rich

micro- and mesopores with a reasonable fraction of micro- and mesopores (1.12) [13]. Therefore, CPAC is a superior adsorbent toward ABX in water and milk [15]. Adsorption of ABX onto ACs is a complex process as it is closely associated with the physicochemical property of ACs, the molecule structure, property and geometric barrier of ABX, and the adsorption conditions, etc. The critical physicochemical properties of ABX, such as electrostatic interaction and Lewis acid-base interaction ($pK_a$), hydrophobicity ($S_{water}$ and $LogK_{OW}$), electronic coupling (the π–π electron donor-acceptor (EDA) interactions and H-bonding), pore filling (molar volume and molecular size), etc., influence the adsorption affinity [27–31].

The major physicochemical properties, chemical structures and the molecular sizes of model ABX are listed in Table S1 and Scheme 1.




SMM OTC

CEF MAR

Scheme 1 Chemical structures and molecular sizes of the model ABX (The molecular sizes were calculated from the Chem3D Program).

As shown in Scheme 1, SMM contains a sulfonamide group, which connects with an aniline ring and an aromatic heterocyclic group. The unionized form of SMM has the smallest molecule size among the four model ABX, while CPAC has a high $V_{Meso}$ and $S_{BET}$, which favor the adsorption of low-sized sulfonamide ABX due to the pore-filling effect [27,31,32]. In addition, the strong electron-withdrawing sulfonamide group causes the decrease of the π-electron density in aromatic rings and the heterocyclic group, and hence act as π-electron acceptors. In contrast, the π-electron-rich regions on the graphene surface of CPAC serve as π-electron donors. Such π-π EDA interactions enhance the adsorption of SMM on CPAC [27,29]. Furthermore, the rich O-containing groups (4.04±0.98%) of CPAC favor the adsorption of relatively hydrophilic SMM [29,30]. Finally, electrostatic interactions and H-bonding may play additional roles [30,33].

OTC belongs to tetracycline ABX, possessing one tetracycline (TC) ring and multiple hydroxyl (-OH), N,N, dimethyl (-$N(CH_3)_2$) and amide (-$C(O)NH_2$) groups on the TC ring. The molecular size of OTC is the second smaller among the four model ABX. Similar to the above, the higher adsorption affinity of OTC onto CPAC may be mainly attributed to the high $V_{Meso}$, $S_{BET}$ of CPAC, and the pore-filling effect [18,34]. In addition, the electrostatic interaction and the π–π interactions between OTC and CPAC should be involved in the adsorption of OTC [35]. The multiple -OH, -$N(CH_3)_2$ and -$C(O)NH_2$ groups in the OTC molecule are electron donors and induce strong conjugation with the π-electrons on aromatic rings and interact electrostatically with the positively charged regions on CPAC. The H-bonding between -OH group and O-

containing functional groups on CPAC is also important due to the appropriate contents of O-containing groups on CPAC. Also, O-containing groups facilitate the adsorption of relatively hydrophilic OTC [27,36].

Compared with the higher adsorption affinity of SMM and OTC on CPAC, the adsorption efficiencies of CEF and MAR onto CPAC are relatively lower (Fig. 2). CEF is the hydrochloride salt of ceftiofur, containing a $\beta$-lactam core structure (a cephem) coupling with a furan-carboxylic-thioester, an amide, an iminomethoxy, and a 2-amino-4-thiazoyl group. CEF has the largest molecular size and the highest hydrophobicity (Log$K_{OW}$: 1.2) among the four model ABX. The large molecular size of CEF causes a significant size-exclusion effect or geometric barrier, reducing the affinity with CPAC [27,31,32,34,37]. Meanwhile, its high hydrophobicity is unfavorable to the complex interactions of CEF with the rich O-containing groups on the surface of CPAC. Finally, acidic groups (–COOH) in hydrophobic compounds negatively influence the adsorption on ACs due to the formation of large and dense water clusters [28,38].

MAR is one of fluoroquinolones, containing a bicyclic core structure related to the substance 4-quinolone, a fluorine atom in the chemical structure, –COOH, 3,5-dimethylpiperazine, carbonyl, etc. CEF has the second larger molecular size and the second higher hydrophobicity (Log$K_{OW}$: -0.5) among the four model ABX. As mentioned above, both larger molecular size and higher hydrophobicity are unfavorable to the adsorption of MAR on CPAC. It is similar to CEF, –COOH group in the MAR may reduce its affinity with CPAC [28,38]. Additionally, F-containing organics may exhibit a weaker affinity with ACs [39].

The effects of eluents on the elution of CEF and MAR from CPAC have been discussed in detail in Section 3.1.2. It can be concluded that the acidity or alkalinity, polarity and H-bond interactions of eluent jointly dominate the elution performance of ABX from CPAC and the co-solvent 30% $NH_4OH$/n-PrOH (1/19 v/v) is an appropriate eluent to elute CEF and MAR from CPAC [8,23,24]. In contrast, 30% $NH_4OH$/EtOH (1/19 v/v) is appropriate to effectively elute SMM and OTC. The prominent difference between these two eluents is the difference in polarities of EtOH and n-PrOH. The ionized SMM at the presence of $NH_4OH$ facilitates its elution by the polar eluent of 30% $NH_4OH$/EtOH (1/19 v/v) based on the like-dissolves-like rule [29]. Similarly, OTC with the strongest polarity among the four model ABX can be effectively eluted by 30% $NH_4OH$/EtOH (1/19 v/v). Meanwhile, the strong H-bond interactions of 30% $NH_4OH$/EtOH enhance the elution of SMM and OTC.

### 3.2. Optimization of AC-SPE for ABX recovery from milk

The major challenge encountered in the analysis of ABX in milk is separating the impurities, e.g. fat, protein, etc., and increasing the concentration of trace ABX [2,5]. Using the optimization of AC-SPE for ABX recovery from water as a base, the technique was further optimized to validate the feasibility of recovering ABX from milk, and this included determining the *REs* of ABX using various adsorbents at different concentrations, determining the limit of detections (LODs), the limit of quantitations (LOQs), the linear ranges (LRs) and errors. Finally, the AC-SPE process was compared to other methods reported for the analysis of model ABX in milk.

### 3.2.1. Recovery of ABX using various ACs at different concentrations

To find an appropriate adsorbent, the *REs* of ABX were obtained at various concentrations in different milk volumes using 50 mg PGAC, 10 mg WPAC or CPAC. 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were used to elute ABX from AC columns. As a result, 0.5 mL HPLC samples were prepared from 10-80 mL of 0.02-1.00 μg/mL medicated milk using AC-SPE; the analytes in milk were concentrated 20-160 fold.

It is difficult for large-volume milk to pass through the powder AC-SPE columns due to the higher viscosity. Therefore, EDTA had previously been added to the milk samples (containing 10 mM EDTA) to ensure the smooth flow of the large-volume milk samples through the CPAC columns [2,5], which are designated as CPAC'. The results of *REs* are summarized in Table 2.

Table 2. *REs* of ABX at various concentrations from milk using different ACs.

| ***ABX*** | ***$C_0$ (μg/mL)*** | ***$V_s$ (mL)*** | ***RE (%) (mean, n=3)*** | | | |
|---|---|---|---|---|---|---|
| | | | **PGAC** | **WPAC** | **CPAC** | **CPAC'** |
| SMM | 0.02 | 80 | - | - | - | 99.48±1.28 |
| | 0.10 | 40 | 4.89±0.06 | 47.82±0.17 | 98.18±1.03 | 99.53±0.51 |
| | 0.50 | 20 | 4.69±0.01 | 45.04±2.55 | 94.89±1.11 | 98.89±0.90 |
| | 1.00 | 10 | 4.24±0.00 | 40.57±2.26 | 94.79±0.68 | 98.59±0.75 |
| OTC | 0.02 | 80 | - | - | - | 99.67±0.42 |
| | 0.10 | 40 | 5.81±0.17 | 49.25±0.54 | 81.33±3.52 | 99.33±0.17 |
| | 0.50 | 20 | 4.49±0.03 | 45.70±0.75 | 79.78±1.7 | 99.30±0.06 |
| | 1.00 | 10 | 4.48±0.11 | 42.10±0.34 | 78.47±2.36 | 99.07±1.34 |
| CEF | 0.02 | 80 | - | - | - | 69.13±1.40 |
| | 0.10 | 40 | 1.16±0.02 | 11.81±0.30 | 38.41±1.26 | 69.01±0.16 |
| | 0.50 | 20 | 1.02±0.03 | 8.11±0.29 | 36.17±0.33 | 68.62±0.32 |
| | 1.00 | 10 | 0.89±0.00 | 8.30±0.02 | 38.36±3.33 | 68.79±0.32 |
| MAR | 0.02 | 80 | - | - | - | 61.93±1.05 |
| | 0.10 | 40 | 1.00±0.01 | 22.80±0.69 | 41.83±2.54 | 61.89±1.19 |
| | 0.50 | 20 | 0.90±0.09 | 21.47±0.19 | 38.65±0.02 | 60.98±0.37 |
| | 1.00 | 10 | 0.90±0.10 | 19.25±0.81 | 37.08±1.20 | 60.81±0.19 |

Adsorption conditions: 10-80 mL of medicated milk were passed through 10 mg-WPAC, CPAC and 50 mg-PGAC columns at 1.00 mL/min at room temperature. Elution conditions: 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were

passed through the ABX-loaded AC columns at 0.07 mL/min at room temperature.

**Note:** $C_0$, initial concentration of ABX; $V_s$, volume of the sample; *RE*, recovery efficiency.

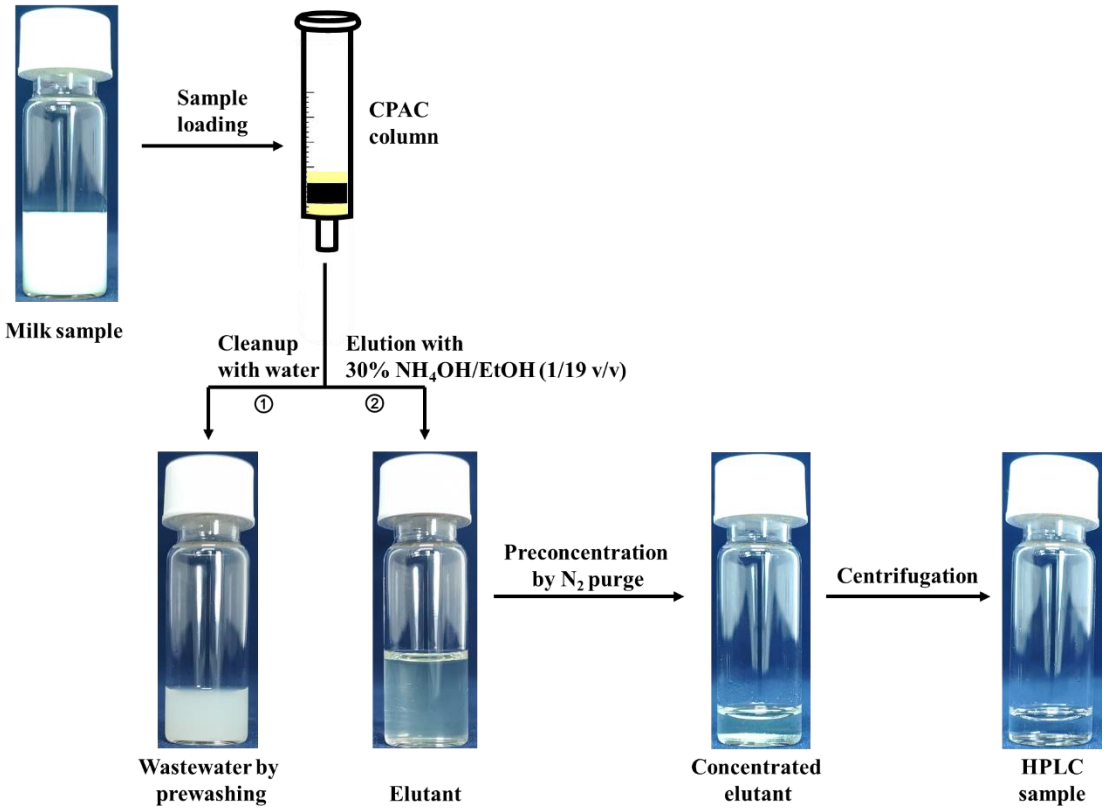
As shown in Table 2, the *RE* ranges of various ABX using CPAC, WPAC and PGAC are 37.6%-96.0%, 9.4%-45.6% and 0.93%-4.93%, respectively. The *REs* positively correlate with the $S_{BET}$ of the ACs; the increasing $S_{BET}$ provides more adsorption sites. The mean *REs* of ABX with CPAC at different concentrations in milk are ordered as follows: SMM (96.0%) > OTC (79.9%) > MAR (39.2%) > CEF (37.6%). The *REs* obtained from the milk samples are somewhat lower than those from the water samples, and this may be the result of the possible chelation of ABX with milk components. As for the differences in the *REs* of various ABX in milk, the order of *REs* is similar to that obtained in water samples and reasons are discussed in Section 3.1.3.

Surprisingly, adding EDTA to the milk samples not only made the milk flow more smoothly, but also significantly improved the *REs* of the model ABX. As listed on CPAC' in Table 2, the mean *REs* with EDTA of SMM, OTC, CEF and MAR at different concentrations are 99.1%, 99.3%, 68.9% and 61.4%, respectively. These are 3.3%, 24.4%, 56.7%, and 83.0% higher, respectively, than those obtained in the absence of EDTA. This remarkable improvement in *REs* may be attributed to the effective separation of ABX/proteins/metal ion (e.g. $Ca^{2+}$) chelates by EDTA [9]. Thus, higher *REs* can be obtained by CPAC-SPE for the analysis of ABX in milk.

Furthermore, 15 mg of CPAC and 30% $NH_4OH$/n-PrOH (1/19 v/v) have been proven to be effective adsorbent dose and eluent for the recovery of CEF and MAR in water samples. Aiming to increase the *REs* of CEF and MAR, 15 mg of CPAC and 30%

496 $NH_4OH$/n-PrOH (1/19 v/v) were used as adsorbent and eluent. Unfortunately, higher
497 *REs* cannot be achieved after unremitting efforts due to the interference of milk
498 components. Hence it is necessary to promote the recovery of CEF and MAR in milk
499 by using AC-SPE method in the future.

500 Besides the preconcentration of ABX from milk, the separation or removal
501 efficiency of major components in milk is another concern. Fig. 6 shows the appearance
502 of the samples during SPE process.




503
504 Fig. 6 Appearance of samples collected during the pretreatment procedure for HPLC analysis.

505 As shown in Fig. 6, most of the white milk components that remained in the AC
506 columns were washed out using 1 mL of distilled water, and very few residual white
507 components were further removed from the elutant via centrifugation.

508 Fig. S1 illustrates a HPLC comparison of samples from blank milk, samples that

were recovered from medicated milk by using 10 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) for elution, as well as standard ABX aqueous solutions. As shown in Fig. S1, only one peak appears for the standard CEF aqueous solution and this is at 12.6 min, while no peak was observed around 12.6 min for the sample from blank milk under the same HPLC conditions. This indicates that milk components did not interference with the CEF analysis. In contrast, the peak for CEF appears at around 12.6 min in the sample recovered from the CEF-spiked milk using CPAC-SPE. In addition, two tiny peaks, at around 7.8 min and 11.8 min, are found, and these probably arise from the impurities in the CEF stock solution. Similarly, the milk components did not interference in the OTC analysis, although three tiny peaks are found in the sample recovered from the OTC-spiked milk. Fortunately, the peaks do not disturb the OTC analysis. The HPLC analyses of MAR and SMM are better still as no problematic peaks are observed in the samples recovered from the MAR- and SMM-spiked milk. Moreover, EDTA did not appear in any HPLCs, which is consistent with a previous study [7]. Overall, the interference of milk components with the HPLC analysis of ABX by using CPAC-SPE can be neglected.

### 3.2.2. Validation of the CPAC-SPE-HPLC method

The European Commission Decision (2002/657/EC) on the performance of analytical methods and the interpretation of results was published in 2002 [40]. The CPAC-SPE-HPLC method for detecting trace ABX in milk was validated, with reference to the above Decision, by investigating LOD, LOQ, LR/$R^2$ (correlation coefficient), reproducibility and intraday/interday precision. Based on the MRLs of

SMM, OTC, CEF and MAR regulated by European Commission [1], LOD and LOQ value for the model ABX were set as 0.020 μg/mL. Therefore, 80 mL of 0.020 μg/mL medicated milk containing 10 mM EDTA were preconcentrated and cleaned up via adsorption with 10 mg-CPAC columns, elution with 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v)), and purging with $N_2$ gas to prepare 0.5 mL of HPLC samples. Hence, the trace ABX was concentrated 160-fold in the HPLC samples, and the *REs* of SMM, OTC, CEF and MAR reached 99.48%, 99.67%, 69.13% and 61.93%, respectively.

In this range of 0.02-2.00 μg/mL of ABX, the linear correlation of ABX concentrations in medicated milk and HPLC samples was investigated using CPAC-SPE. The high $R^2$ values (0.993-0.998) for the four model ABX indicate that the linear correlation between ABX concentrations in medicated milk and HPLC samples is superior. The reproducibility of CPAC-SPE-HPLC was investigated in six repeated tests. Reproducibility is expressed as the relative standard deviation (RSD, %) and shown in italics in parentheses in Table 3.

Table 3. Mean *REs* (%) (n = 6) of ABX in milk using CPAC-SPE-HPLC, and reproducibility (RSD, %).

| $C_{ABX}$ (μg/mL) | SMM | OTC | CEF | MAR |
|---|---|---|---|---|
| 0.02 | 99.70 (*6.3*) | 99.93 (*6.5*) | 69.69 (*4.0*) | 61.69 (*2.4*) |
| 0.10 | 99.61 (*2.4*) | 99.61 (*6.4*) | 69.28 (*3.2*) | 61.72 (*2.0*) |
| 0.50 | 99.48 (*4.0*) | 99.76 (*3.1*) | 68.84 (*2.2*) | 60.58 (*2.2*) |
| 1.00 | 99.66 (*5.1*) | 99.23 (*3.2*) | 68.16 (*3.7*) | 61.02 (*1.2*) |

**Note:** $C_{ABX}$, initial concentration of ABX in milk.

As listed in Table 3, the RSDs are in the range of 2.4%-6.3%, 3.1%-6.5%, 2.2%-4.0%, and 1.2%-2.4% for SMM, OTC, CEF and MAR, respectively, demonstrating that CPAC-SPE-HPLC has good determination reproducibility.

Furthermore, the precision of this method was investigated in terms of intraday and interday assays. The intraday/interday precision of CPAC-SPE-HPLC was evaluated using 0.1 μg/mL and 1.0 μg/mL medicated milk, which was consecutively tested five times in the same day (n = 5) and also over three different days (n = 15). For 0.1 μg/mL ABX in milk, the intraday RSD values obtained ranged from 1.2% to 6.4%, whereas the interday RSD values were in the range of 5.5–8.3%. For 1.0 μg/mL ABX in milk, the intraday RSD values obtained ranged from 0.5% to 5.1%, while the interday RSDs were slightly higher, ranging from 4.5% to 7.8%.

### 3.2.3. Comparison with previous analytical methods

The developed CPAC-SPE-HPLC method was compared with previous SPE methods for the determination of trace SMM, OTC, CEF and MAR in milk. The sample-preparation procedure, amount of adsorbent, the volume of eluent, analytical instrument, *REs*, LOD and LOQ of each method are summarized in Table S3 and discussed in Section S2.0.

As seen in Table S3, the higher *RE* of trace SMM and OTC in milk was achieved in this work over the previous SPE methods. The LOD and LOQ values for OTC (0.02 μg/mL) are comparable with the previous data, but the previous methods possessed lower LOD and LOQ for SMM. More importantly, the LOD and LOQ of SMM and OTC (0.02 μg/mL) obtained in this work were lower than their MRLs (0.10 μg/mL). In comparison, the *REs* of CEF and MAR obtained in this work are lower than those reported in previous studies. Due to using higher amounts of adsorbents (200-500 mg), the previous LOD or/and LOQ of CEF and MAR are significantly lower. In contrast,

573 the LOD and LOQ of CEF and MAR (0.02 μg/mL) obtained with 10-15 mg CPAC in
574 this work are still lower than the MRL (0.10 or 0.075 μg/mL).

575 Overall, the simplified sample-preparation procedure for the analysis of trace ABX
576 in milk not only favors increasing *REs*, but also reduces the consumption of solvents
577 and other materials, time and energy. AC-SPE-HPLC possesses inherent advantages,
578 including the wide availability of powder AC sources, low consumption and costs,
579 convenient operation, reliability and superior reproducibility, etc. Besides, the AC-
580 SPE-HPLC method can be widely applied and easily verified and any commercial SPE
581 cartridges are not required.

582 ## 4. Conclusions

583 A simple, accurate, reliable, and miniaturized CPAC-SPE-HPLC method has been
584 successfully developed for the effective preconcentration, cleanup and determination
585 of SMM, OTC, CEF and MAR in milk. Superior *REs* (>99%) of the model ABX in
586 water were obtained in preliminary study. Subsequently, the conditions were further
587 improved to obtain higher *REs* of the four model ABX in milk. 10 mg of CPAC and 2
588 mL of 30% $NH_4OH$/EtOH (1/19 v/v) were scanned as the appropriate adsorbent and
589 eluent. The mean *REs* with EDTA of SMM, OTC, CEF and MAR at different
590 concentrations are 99.1%, 99.3%, 68.9% and 61.4%, respectively. The AC-SPE-HPLC
591 method exhibits a good inter/intraday precision. To further improve AC-SPE-HPLC
592 analysis of ABX in milk and expand the range of applications (water and food), in
593 future, more species of ACs, such as ACs derived from different precursors, modified
594 ACs, magnetic ACs and carbon composites, should be applied and evaluated as

595 adsorbents for SPE pretreatment.

596

597

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# Declaration of interest

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

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# Determination of trace antibiotics in water and milk via preconcentration and cleanup using activated carbon

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Abbreviations: ABX, antibiotics; SMM, sulfamonomethoxine sodium; OTC, oxytetracycline; CEF, ceftiofur hydrochloride; MAR, marbofloxacin; MRLs, maximum residue limits; HPLC, high pressure liquid chromatography; SPE, solid phase extraction; RE, recovery efficiency; ACs, activated carbons; CPAC, coconut powdered AC; $S_{BET}$, Brunauer-Emmett-Teller surface area; $V_{Meso}$, mesopore volume; $V_{Micro}$, micropore volume; PGAC, peat granular AC; WPAC, wood powder AC; MeCN, acetonitrile; TFA, trifluoroacetic acid; MeOH, methanol; EtOH, ethanol; $NH_4OH$, ammonia solution; EDTA, ethylenediaminetetraacetic acid disodium salt hydrate; t-BuOH, t-butanol; n-PrOH, n-propanol; MW, molecular weight; MV, molar volume; Log$K_{OW}$, octanol-water partition coefficient; $S_{Water}$, solubility of ABX in water; $S_{EtOH}$, saturated mole fraction solubility of ABX in EtOH; p$K_a$, dissociation constant; NHA, number of H-bond acceptors; NHD, number of H-bond donors; Refs., references; *Bp*, boiling point; $\varepsilon$, dielectric constant; EDA, $\pi$–$\pi$ electron donor-acceptor; TC, tetracycline; LODs, limit of detections; LOQs, limit of quantitations; LRs, linear ranges; RSD, relative standard deviation; FA, formic acid; DCM, methylene chloride; MSPD, magnetic solid phase dispersion; MSPE, magnetic solid phase extraction.

## S1.0 The information of solvents and water

Acetonitrile (MeCN, ≥99.9%), trifluoroacetic acid (TFA, ≥99%) and methanol (MeOH, 99.9%) were obtained from Sigma-Aldrich (France). Ethanol (EtOH, ≥99.8%) was provided by Sigma-Aldrich (UK). Acetic acid (HOAc, 100%) was purchased from VWR International (European Commission). The ammonia solution (30% $NH_4OH$) was provided by CARLO ERBA Reagents S.r.l. (Italy). Ethylenediaminetetraacetic acid disodium salt hydrate (EDTA, >99%), n-butanol (n-BuOH, >98%), t-butanol (t-BuOH, 99%), and n-propanol (n-PrOH, 99.8%) were purchased from Alfa Aesar (Thermo Fisher Scientific, Germany).

Milli-Q water was obtained from a Milli-Q Reference A+System (Merck Millipore, Darmstadt, Germany) and used for the preparation of HPLC mobile phases. Deionized water (conductivity ≤ 2 μS/cm) was used to prepare standard solutions of ABX, which were refrigerated at 4 °C.

**S2.0 Comparison with previous analytical methods**

All of the samples prepared using SPE have undergone analysis with HPLC, LC-MS or -MS/MS, or UPLC-MS in the past. The present protocol exhibited higher *RE* than the previous SPE methods for the analysis of trace SMM in milk [45,46], as can be seen in Table S3. The previous methods possessed lower LOD and LOQ since high amounts of adsorbents (80-500 mg) were used. In this work, only 10 mg of CPAC was used as the adsorbent and the LOD and LOQ values of SMM (0.02 μg/mL) are also lower than the MRL (0.10 μg/mL). In addition, toxic solvents, such as DCM, MeCN and MeOH, and chemical deproteinization were not required in this study. Similarly, we achieved a higher *RE* for OTC here than the two previous SPE methods [11,47]. The LOD and LOQ values for OTC (0.02 μg/mL) are comparable with the previous data and are also lower than the MRL (0.10 μg/mL), although less CPAC (10 mg) was used as an adsorbent here, as 50 mg of adsorbent was used in the previous method. In addition, chemical deproteinization and redissolution were not required in this study. In terms of toxicity, the use of EtOH as an eluent in this study is better than the MeOH that was used in other studies.

The *RE* of CEF (68.9%) that was obtained using a 10 mg-CPAC column in this work is comparable with that (69.0%) obtained using a 500 mg-Carbograph 4 cartridge-SPE in a previous study [48]. However, the previously reported LOQ is significantly lower than the one in that study since very different amounts of adsorbents were used. Compared with the *RE* (97.0%) obtained in another study using 200 mg-Oasis HLB-MSPD, the *RE* of CEF in this work is significantly lower, but the LOD and LOQ of

CEF (0.02 μg/mL) are comparable with the previous data, and are also lower than the MRL (0.10 μg/mL), although less CPAC (10 mg) was used as the adsorbent [49]. A further benefit is that toxic solvents, such as DCM, MeCN and MeOH, and the redissolution of analytes are not required in this study. The *RE* of MAR (61.4%) that was obtained using a 10 mg-CPAC column in this work is considerably lower than those (82.5%-106.5%) obtained in three previous studies. Moreover, the previous methods had lower LOD and LOQ, but 0.020 μg/mL of LOD or LOQ in this work is still sufficient to meet the requirements for the MRL of MAR (0.075 μg/mL). However, a remarkably lower amount of adsorbent (10 mg of CPAC) was used in this study than the 200-500 mg of adsorbents used in the other studies [25,50,51]. Furthermore, the toxic solvent MeOH and chemical deproteinization were not required in this study.

## Figure S1

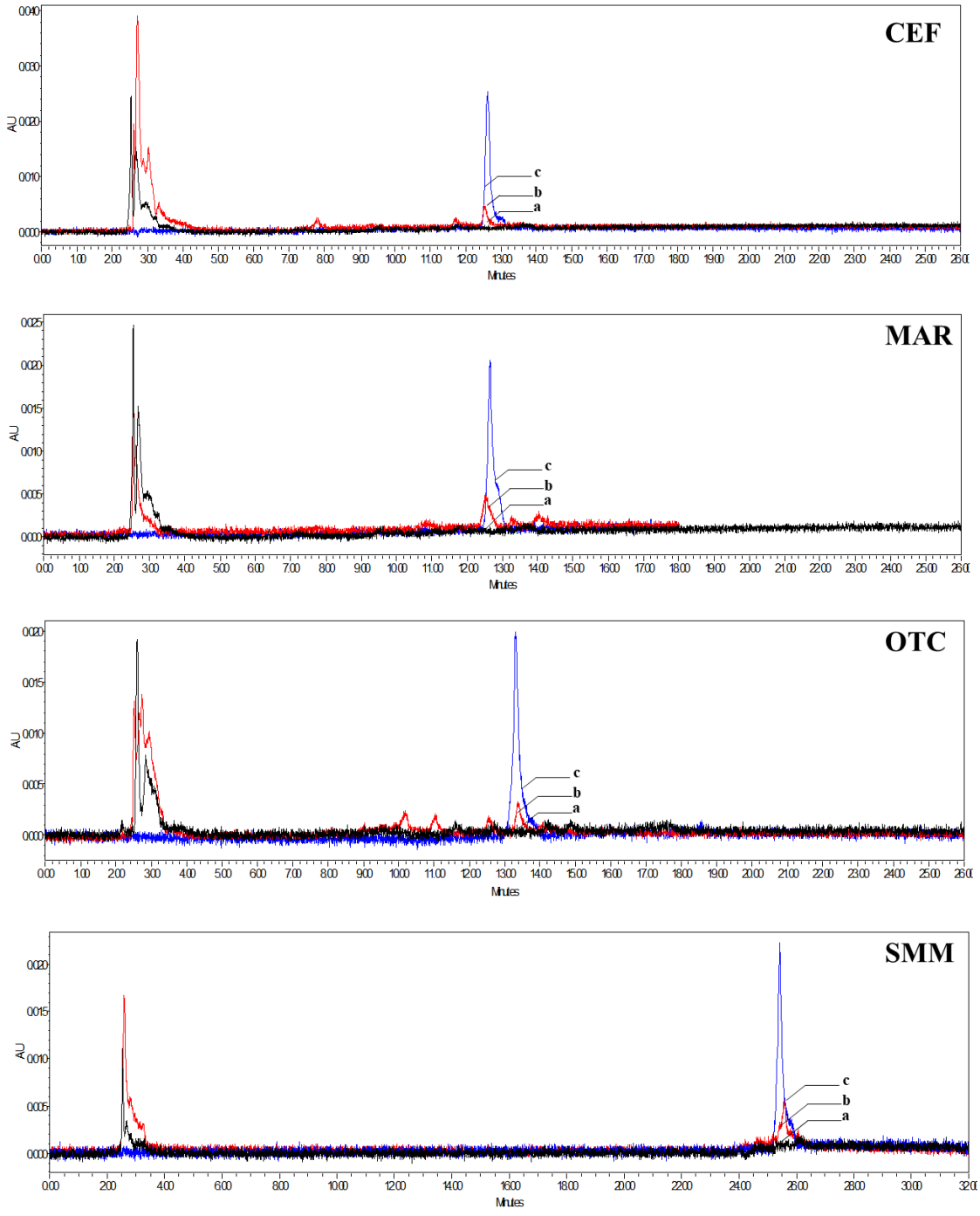


Fig. S1. Comparison of HPLCs from various samples. (a) sample from blank milk, (b) sample recovered from medicated milk by using 10 mg of CPAC for adsorption and 2 mL of 30% $NH_4OH$/EtOH (1/19 v/v) for elution, (c) standard ABX aqueous solutions (50 μg/mL MAR, 100 μg/mL CEF, OTC or SMM).

## Table S1

Table S1. Major physicochemical properties and toxicological parameters of the model ABX (Part of the data was adopted from the Pubchem and Drugbank databases).

| *ABX* | *CAS* | *MW* (*g/mol*) | *MV* ($cm^3/mol$) | *LogK*$_{OW}$ | *S*$_{Water}$ (*mg/L*) | *S*$_{EtOH}$ ($10^3$) | *pK*$_a$ | *NHA* | *NHD* | *MRLs* (*μg/L*) | *LD*$_{50}$ (*mg/kg*) | *Refs.* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SMM | 38006-08-5 | 302 | 213.3 | -0.8 | 10000 | - | 6.33 | 8 | 2 | 100 | 5620 Rat oral | [15,41] |
| OTC | 2058-46-0 | 460 | 270.3 | -0.9 | 300 | [a]12.0 | 3.27 | 11 | 8 | 100 | >2000 Colinus virginianus oral | [15,42,43] |
| CEF | 103980-44-5 | 560 | 290.9 | 1.2 | 100 | 23.3 | 2.83 | 12 | 4 | 100 | - | [15] |
| MAR | 115550-35-1 | 362 | 226.4 | -0.5 | 2600 | 116.6 | 5.38 | 8 | 1 | 75 | >0.002 Mouse oral | [15,44] |

[a] The unit is mg/L; [b] the unit is mg/L/4 hr.

**Note:** *MW*, molecular weight; *MV*, molar volume; *LogK*$_{OW}$, Octanol-water partition coefficient; *S*$_{Water}$, solubility in water; *S*$_{EtOH}$, saturated mole fraction solubility of ABX in EtOH, calculated according to the literature [44]; *pK*$_a$, dissociation constant; *NHA*, number of H-bond acceptors; *NHD*, number of H-bond donors; *MRLs*, maximum residue limits; *LD*$_{50}$, amount of a material, given all at once, which causes the death of 50% (one half) of a group of test animals; *Refs*., references.

## Table S2

Table S2. The HPLC conditions for the determination of the model ABX.

| *ABX* | *Wavelength (nm)* | *Retention time (min)* | *Running time (min)* | *Mobile phase* | |
|---|---|---|---|---|---|
| | | | | *Phase A (%) 0.1%TFA in $H_2O$* | *Phase B (%) 0.1%TFA in MeCN* |
| SMM | 273 | 25.483 | 0 | 99 | 1 |
| | | | 25 | 0 | 100 |
| | | | 32 | 0 | 100 |
| OTC | 267 | 13.313 | 0 | 98 | 2 |
| | | | 25 | 0 | 100 |
| | | | 26 | 0 | 100 |
| CEF | 265 | 12.510 | 0 | 98 | 2 |
| | | | 5 | 70 | 30 |
| | | | 20 | 0 | 100 |
| | | | 26 | 0 | 100 |
| MAR | 298 | 14.018 | 0 | 98 | 2 |
| | | | 25 | 0 | 100 |
| | | | 26 | 0 | 100 |

## Table S3

Table S3. Comparison of the proposed CPAC-SPE-HPLC method with previous SPE methods for the determination of the model ABX in milk.

| *ABX* | *Sample-preparation procedures* | *Eluent* | *Analytical instrument* | *RE* (%) | *LOD* (μg/mL) | *LOQ* (μg/mL) | *Refs.* |
|---|---|---|---|---|---|---|---|
| SMM | Agitation, 500 mg-carbograph 4 cartridge-SPE, redissolution, filtration. | 1.5 mL MeOH-6 mL DCM/ MeOH (80/20 v/v) acidified with 10 mM TFA continuously | LC−ES −MS | 97.3 | [a]0.001 | [a]0.003 | [45] |
| | Deproteinization, 80 mg-magnetic graphene oxide nanocomposite -MSPE, evaporation, redissolution. | 1.0 mL MeCN containing 5 % $NH_4OH$ (v/v) | HPLC | 86.8 | 0.00008 | 0.00025 | [46] |
| | 10 mg-CPAC-MSPE, concentration by $N_2$, centrifugation. | 2 mL 30% $NH_4OH$/EtOH (1/19 v/v) | HPLC | [b]99.1 | 0.02 | 0.02 | This study |
| OTC | Deproteinization, desolvation, redissolution, filtration, 50 mg-molecularly imprinted poly (methacrylic acid) –silica hybrid composite -SPE. | 2 mL MeOH/ HOAc (60/40 v/v) | LC–MS/MS | [b]99.2 | 0.014 | - | [11] |
| | Deproteinization, $C_{18}$-SPE, constant volume. | 0.75 mL MeOH | UPLC-MS/MS | [b]98.8 | - | - | [47] |
| | 10 mg CPAC-SPE, concentration by $N_2$, centrifugation. | 2 mL 30% $NH_4OH$/EtOH (1/19 v/v) | HPLC | [b]99.3 | 0.02 | 0.02 | This study |
| CEF | 500 mg-carbograph 4 cartridge-SPE, desolvation by $N_2$, redissolution, filtration. | 6 mL DCM/ MeOH (80/20 v/v) acidified with 50 mM FA | HPLC | 69.0 | - | 0.001 | [48] |
| | 200 mg-Oasis HLB- MSPD, evaporation to dryness, redissolution. | 1 mL MeOH –2 mL MeCN continuously | HPLC | 97.0 | [a]0.0118 | [a]0.0357 | [49] |
| | 10 mg-CPAC-SPE, concentration by $N_2$, centrifugation. | 2 mL 30% $NH_4OH$/EtOH (1/19 v/v) | HPLC | [b]68.9 | 0.02 | 0.02 | This study |
| MAR | Centrifugation, PLRP-cartridges-SPE. | 9 mL $H_2O$/MeOH ( 80/20 v/v) plus 0.5% FA (1.5 mL) | HPLC | 106.5 | 0.0001 | - | [50] |
| | Deproteinization, filtration, redissolution, centrifugation, 500 mg-Oasis HLB cartridges-SPE, evaporation to dryness, redissolution. | 10 mL MeOH containing 1.5% (w/v) HOAc | LC-MS | [b]100.5 | 0.00009 | 0.00031 | [51] |
| | Deproteinization, 200 mg-ionic liquid based chitosan-SPE, desolvation by $N_2$, constant volume. | 5 mL 20% $NH_3$ (v/v MeOH) | LC–MS/MS | 82.5 | 0.00423 | - | [25] |
| | 10 mg-CPAC-SPE, concentration by $N_2$, centrifugation. | 2 mL 30% $NH_4OH$/EtOH (1/19 v/v) | HPLC | [b]61.4 | 0.02 | 0.02 | This study |

[a] The unit: ppm; [b] with EDTA in medicated milk.

**Note:** *RE*, recovery efficiency; *LODs*, limit of detections; *LOQs*, limit of quantitations; *Refs*, references; *FA*, Formic acid; *DCM*, Methylene chloride; *MSPD*, Magnetic solid phase dispersion; *MSPE*, Magnetic solid phase extraction.

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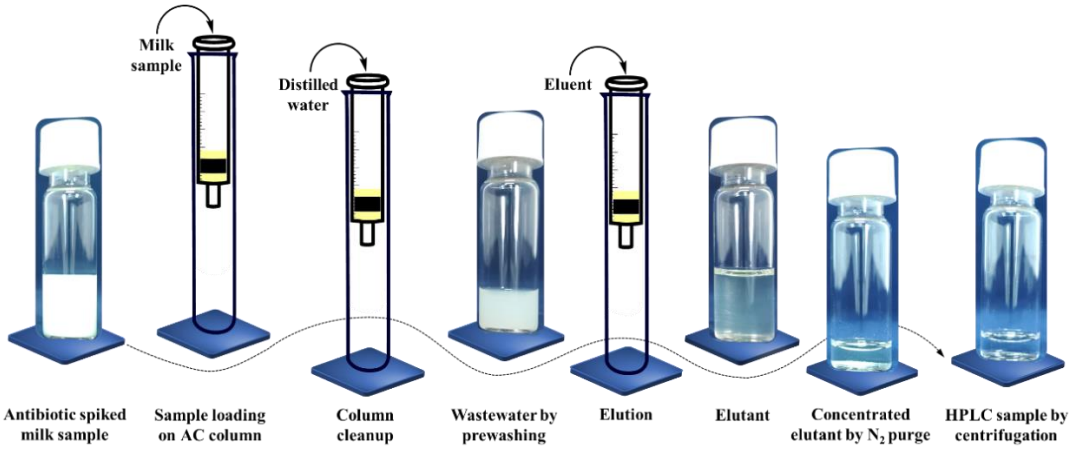
## Graphical abstract




Simple preconcentration and cleanup of trace antibiotics in milk using activated carbon (AC)-based solid phase extraction for HPLC analysis.